

CHEMICAL ASPECTS OF ECOLOGY IN RELATION TO AGRICULTURE

Hubert Martin

CANADA DEPARTMENT OF AGRICULTURE
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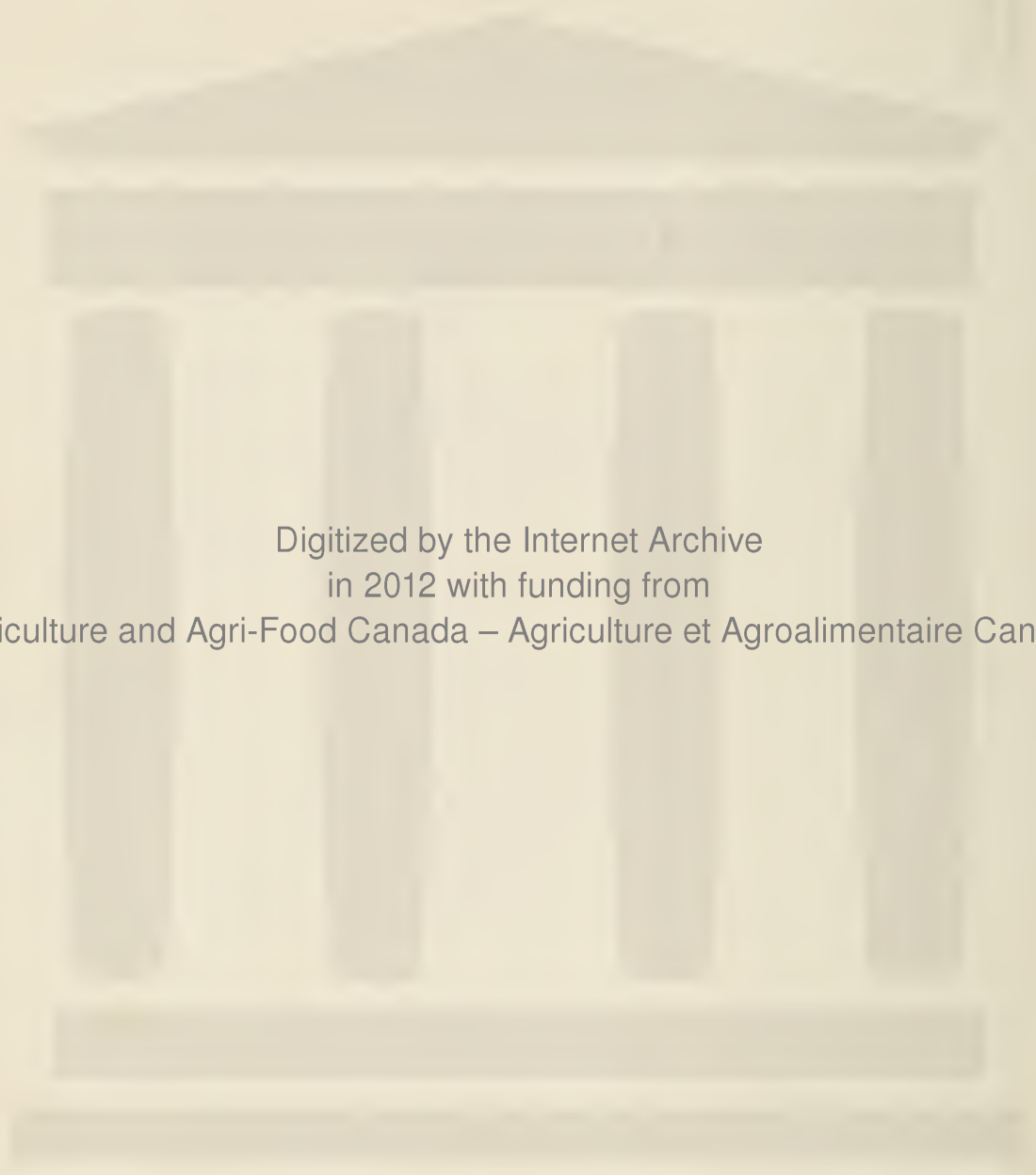
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CHEMICAL ASPECTS OF ECOLOGY IN RELATION TO AGRICULTURE

by Hubert Martin

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CONTENTS

CHAPTER	PAGE
1. Introduction	5
2. The production of phytotoxins by higher plants	6
3. Higher plants vs. insects: being mainly concerned with the chemical defenses of plants against insects	22
4. Higher plants vs. fungi: the chemical defenses of plants against fungal attack	33
5. Higher plants vs. bacteria: being mainly concerned with the rhizosphere	38
6. The reaction of higher plants to insect attack	40
7. Fungi vs. higher plants: the role of phytotoxins in plant pathology	43
8. Fungi vs. fungi: the chemical basis of biological control	53
9. The production of insecticides and insect attractants by insects and microorganisms	67
10. Bacteria vs. higher plants	73
11. The ecological chemistry of bacteria	77
Author index	88
Subject index	93

Chapter 1

INTRODUCTION

In 1950, the Canada Department of Agriculture established, on the campus of the University of Western Ontario, a laboratory whose main task is the study of the ecological consequences of the widespread and continued use, in agriculture, of chemicals of such biological activity that they serve for the control of the pests, diseases, and weeds of crops. In conjunction with that study, it was necessary to examine the available information on the biologically active chemicals already present in the crop environment and involved in the interplay of host plant, pest, pathogen, and weed. A knowledge of these chemicals is prerequisite to a study of the effects of the exotic chemical introduced into that environment as insecticide, fungicide, or herbicide.

The science and practice of crop husbandry reduces ultimately to the proper utilization of natural resources for the protection of the crop from factors, biological or otherwise, that tend to depress yield. The interplay of the biological factors is frequently regarded as a competition and at one time it was held that competition between individual plants of the crop was solely for nutrient, light, and water. But many examples are now known of the production of biologically active diffusates from the roots of growing plants. The attack of most, if not all, fungal and bacterial pathogens involves the production of phytotoxins. The specificity so often shown by insects in their range of host plants is often susceptible to biochemical explanation. Moreover, soil fertility is the resultant of a complex interplay of soil fauna and microflora, and it is known that many soil organisms are capable of producing metabolic products of such intense biological activity that they are termed antibiotics. Antibiosis and symbiosis are therefore of basic importance in agriculture for they control the detail of the ecological community of the crop.

The purpose of this report is to review and, as far as possible, classify and correlate information concerning the significance and nature of the chemical factors involved in this antibiosis and symbiosis. It is convenient to catalogue this information into chapters based on the main biological components of the crop environment—higher plants, insects, fungi, bacteria—in their intra- and inter-group relationships.

Chapter 2

THE PRODUCTION OF PHYTOTOXINS BY HIGHER PLANTS

Competition for the available light, air, water, and nutrient is the predominant factor limiting plant populations, whether the plants are growing as crops in temperate climates, as communities of different species in grassland or forest, or under natural conditions. Climate and soil factors determined by climate and geology will decide the natural vegetation. It is usually accepted that these factors are sufficient to account for the numbers and species of the plant community, for plant associations, and for their interactions. "Competition is purely a physical process . . . Two plants, no matter how close, do not compete with each other as long as the water content, the nutrient material, the heat and light are in excess of the needs of both." This was Clements' statement in 1907 (25).

So downright a dictum reflects the doubts current at that time on the possibility that plants may by other means affect the growth of their competitors. Alternative factors had been sought not only because of the suspicion that nature abhors simplicity but because of the many mechanisms, such as excretion of toxins, by which a particular plant species could secure an advantage over rival species. The operation of such processes had frequently been suspected by early natural historians. John Evelyn, in his paper presented to the Royal Society on April 29, 1675, wrote (and I quote from p. 23 of his discourse entitled "*Terra*" published as an appendix to "*Silva*", 5th ed., London, 1729):—"Moreover, Ground is sometimes barren, and becomes unfruitful by the Vicinity of other *Plants*, sucking and detracting the Juice of the *Earth* from one to another: For thus we see the *Reed* and *Fern* will not be made to dwell together; *Hemlock* and *Rue* are said to be inimicous; the *Almond* and the *Palm*, which are seldom fruitful but in Conjugation; and perhaps there are *Effluvia*, or certain inconspicuous *Streams* of dusty *Seeds*, which not only impregnate *Places* where never grew any before, but issue likewise from one to another, as in our *Junipers* and *Cypress* I observe, flowering about April; which are *Trees* of Consort, and thrive not well alone. The *Ficus* never keeps her Fruit so well, as when planted with the *Caprific*. By what Irradiations the *Myrtle* thrives so with the *Fig*; the *Vine* affects the *Elm* and *Olive* (which is at Antipathy with the *Oak*, and imparts also such a Bitterness to the Mould, as kills *Lettuce* and other subnascent *Plants*) is hard to say; and why some affect to live in Crouds, other in Solitude:"

Evelyn was not unmindful of the infertility which results from exhaustion of nutrients, for in the same lecture (*loc. cit.*, p. 29) he says: "All *Plants* do not easily become *Denizons* in all *Places*: I might add to this the Niceness of their *Palates*, and Fondness to their own *Homes*, and to live some in *Consort*, some in *Solitude*, some on dry *Banks*, some in watery *Puddles*, and some as it were in the very *Air*, and fiery *Soils*; nay, some which are found to destroy the vegetable *Virtue* where they grow; for such are said to be *Woad*, *Hemp*, the *Scythian Lamb* (see page 11), etc. and if it be true and constant, all our inbibitions of *Salts* and *Composts* signify little to *Earth* pre-impregnated with a *Salt* or *Virtue* different from what the *Plant* does naturally delight in,

some obscure Footsteps of which every *Ploughman* seems to discover, which makes him change the Crop in some Places yearly: For the first, second and third Burden of the same Grain, especially *Wheat*, will exhaust that which is its proper Aliment . . . ”

Evelyn refers in the final sentence of this quotation to the practice of crop rotation, and perhaps the first scientific approach to the reasons for the success of this practice is due to de Candolle. He was impressed by the evidence he accumulated that the roots of many plant species excrete substances obnoxious to other plants. In Book 5, Chapter 15, S-5, of his great work, “*Physiologie Végétale*” (Paris, 1832) he wrote on page 1474: “MM. de Humboldt et Plenck ont eu l'idée ingénieuse de chercher dans ce fait [plant excretions] la cause des attractions et des répulsions de certaines plantes. Ainsi, si, comme on l'admet assez généralement, le cirse des champs nuit à l'avoine, l'euphorbe et la scabieuse au lin, l'érigeron acre et l'ivraie au froment, etc., cela pourrait être dû à ce que ces plantes suintent par leurs racines quelque chose de nuisible à la végétation des autres: si, au contraire, la salicaire se plaît auprès du saule, la truffe auprès du chêne ou du charme, etc., c'est que peut-être elles tirent parti de quelque excrétion des racines de ces végétaux.” And, later, on page 1496, he wrote: “Ainsi, un pêcher gâte le sol pour lui-même, à ce point que, si, sans changer la terre, on replante un pêcher dans un terrain où il en a déjà vécu un autre auparavant, le second languit et meurt, tandis que tout autre arbre peut y vivre.”

It is not surprising, therefore, that in his chapter on Crop Rotation he assigned great importance to the role of root excretions, and stated that the theory of crop rotation rests on the fundamental fact that plants thrive poorly on soil that has just been cropped with the same species, genus, or family. He distinguished between ‘l'épuisement du sol’, which is the improverishment of the soil arising as a non-specific consequence of the crop on vegetation in general, and ‘l'effritement du sol’, a specific corruption of the soil by dangerous materials such as root excretions. De Candolle foresaw that, if this theory is correct, it may also explain the favorable effect that leguminous crops may exert on succeeding cereal crops.

The refutation of de Candolle's hypothesis of ‘l'effritement du sol’ as the only explanation for the success of crop rotation was easy. Boussingault, in 1838, found in his pot experiments that peas and clover, unlike wheat, apparently acquire nitrogen from the air. The presence of the nodules on the roots of legumes had long been recognized, and Malpighi had, in 1679, described them in his *Anatomia Plantarum* as galls caused by insects. Lackmann (50) recognized that the nodules contain “vibro-like” organisms. The conclusive answer was obtained by Hellriegel whose work was published, with Wilfarth (40) as co-author, in 1888. The “favourable root excretion” became the nitrogen-fixing activity of bacteria, species of the genus *Rhizobium*, when growing in symbiosis with the legume.

An example of similar specificity, but from the opposite side, is the evil effect that barberry exerts on wheat—an effect known from the earliest days. De Candolle devoted a whole section of his book to this topic, but did not include it among the examples on which he based his root excretion hypothesis. Being apparently unaware of the demonstration by Schoeler in 1818 of the connection between barberry and wheat rusts, he dismissed the possibility that the aecidiospore from barberry could, falling on wheat, produce “la rouille”, the cause of which he knew as “l'Uredo”. He favored rather the idea that the pollen of the barberry in some way rendered the wheat flower sterile, and outlined several tests of this hypothesis concerning the influence of one

plant upon another. But it was unnecessary to follow up his suggestions, because De Bary in 1856 established the existence of heteroecism in the rust fungi.

Returning to the general hypothesis of crop rotation, the next development was due mostly to Justus von Liebig (52). He applied his ideas on the mineral nutrition of plants with such success that a general acceptance followed his view that the merit of crop rotation was its balanced utilization of the reserves of inorganic plant nutrients. Support for this idea was early supplied by the work of Daubeny (27), but from time to time doubts have since been mooted that Liebig's hypothesis is not a complete explanation.

In the first decade of the 20th century, the United States Department of Agriculture was faced with the problem of the continually decreasing yield of wheat—even when apparently well cultivated and manured. It was often the sole crop of the prairies. Milton Whitney, Chief of the Bureau of Soils, wrote in 1909 (90): "We know that plants do emit organic substances which are deleterious to themselves". To demonstrate his point he took a small pot holding a pound of soil and in it grew six wheat plants. After three weeks growth he cut the plants at soil level and sowed six more wheat grains. The second crop gave but half the yield of the first plants. Moreover, he cited the following experiment made at Cornell. At one end of a long box corn was planted, at the other end weeds. If a partition was placed between corn and weeds both grew normally, but in the absence of a partition, and when the roots of the crops were allowed to intertwine, the corn refused to grow. Whitney went on, "The reason we keep weeds out of crops, especially during the early period of growth, is not that they affect the moisture content, although they may do so, nor is it that they take up plant food, but that their presence is obnoxious and apparently poisonous to the crop".

Support for Whitney's statements was forthcoming from the work of Schreiner and his colleagues of the Bureau of Soils (77) who had isolated, from unproductive soils, a range of phytotoxic organic compounds. These compounds included protein degradation products, a pentosan, several coumarins and, in particular, a dihydroxystearic acid. These compounds were thought to arise not only from the growing crop, but from plant remains by microbial action. Whatever the source, Cameron (23) wrote: "It is beyond dispute, however, by reason of a large and increasing weight of evidence, much of it direct experiment, that, as a result of the growing of plants, soils and the soil water do contain organic substances harmful to the plant or organism eliminating them; harmful, innocuous or even stimulating to other plants or organisms".

The views of the Bureau of Soils were never widely accepted; they were at once contested by Russell (74). He pointed out that the assessment of phytotoxicity by water-culture techniques, as used in the American experiments, may be misleading—for if these techniques are applied to soil, sorption by soil colloids might rapidly remove compounds that proved toxic in water culture. The efficiency of the colloidal matter of soil in removing, by adsorption, biologically active compounds from the soil solution is a factor that reappears continually in ecological chemistry. Clearly the significance of this factor will depend on the nature of the chemical and the type of soil under study; thus a ready adsorption of surface-active or volatile compounds could be expected. A strong degree of adsorption would also be expected in a muck soil rich in organic matter and, indeed, as the workers of the Bureau of Soils showed, the effects of toxin may often be mitigated by a suitable manuring. A particular answer to Russell's contention is, however, provided by the work of Skinner (78). He showed that aldehydes, including formaldehyde, salicylaldehyde, and vanillin, are toxic to plants growing in culture solutions, that

vanillin and salicylaldehyde occurred in soils of low productivity, and that their harmful effects are reduced by liming or by phosphate application. Moreover, when these aldehydes are applied to soil in quite heavy amounts of 200-400 lb. per acre, toxic effects appeared in plants grown in seven of the twelve soils treated. From the seven soils the aldehyde could be recovered at the end of the growing period, whereas none could be recovered from the "good" soil in which the plants remained healthy. Decomposition, and not adsorption, seems to be the reason for the removal of the toxin.

Russell and others have advanced alternative explanations for the reduction of yield that may follow continuous cropping. It was shown that the plowing in of stubble, particularly of sorghum, immediately reduces the nitrogen available to the crop; Hartland and his colleagues at Rhode Island (40) associated the lowered productivity of an unsuccessful rotation with change of soil acidity. Soil sickness could often be explained by the accumulation of disease favored by monoculture. Similarly, in one-crop successions, particular weeds will become more difficult to eradicate and control; the formation of a plow "hard pan" may interfere with soil drainage and aeration. Loehwing (54) reviewed the literature of the problem up to 1937, which he summarizes with the statement that many authorities accept the conviction of Russell "that there is no evidence of plant excretions conferring toxic properties on the soil". And Russell's words had weight, for in his hands was the control of plots of long years of continuous cropping at the Rothamsted and Woburn Experimental Stations.

The experience of continuous cropping at Rothamsted, Woburn, and elsewhere, has shown that except for leguminous crops it is unnecessary to accept de Candolle's toxin hypothesis to explain the benefits of crop rotation. Ripley (72) who, in his report of long-term Canadian work on crop rotation, gave a full review of the earlier work on toxin hypotheses, found no need to use these ideas in discussing the results of the Canadian work.

Although the testing of an apparently unnecessary hypothesis is unattractive to most scientists, Mann and Barnes (55-59) of the Woburn Experimental Station have continued this study and it is pertinent to review their results in some detail. The prevalent annual weeds of barley grown continuously at the Station since 1877 are spurry (*Spergula arvensis* L.) and scentless mayweed (*Matricaria inodora* L.). They concluded that the effect of these weeds on barley was the result only of competition either for root space or for nutrient when nitrogen is not present in excess. Chickweed (*Stellaria media* (L.) Cyrillo) reduced barley yields though barley had little effect on chickweed—a differentiation explained by the more rapid development of chickweed roots. The roots of these species intertwine freely, and there is no evidence of any specific effect. But when the weed is a grass, results are otherwise. The presence of the twitch grass *Holcus mollis* L. halved the growth of barley when the plants were grown so thinly that competition for root space was abolished; under like conditions, barley reduced by about 75 per cent the growth of the twitch grass. Mann and Barnes wrote: "there seems no doubt of the effect of one plant on the other even though they are grown in the presence of ample supplies of water, plant food, etc., and under conditions when no competition for root space can occur". Continuing their experiments using another twitch grass *Agrostis gigantea* Roth. they concluded that the injurious effect of one grass on another seems to be proved, and that the effect varies from one grass to another and seems to be a specific property of each grass.

An earlier report of the excretion of toxic material by growing couch grass was given by Ostwald (66). Dried and ground roots of *Agropyron repens* (L.) Beauv. were extracted with water and the extract, after autoclave sterilization, was found to inhibit the germination of "seeds" of dandelion (*Taraxacum*). The improved extraction by aqueous ammonia indicated that the toxic substance is acidic; it is also soluble in water and alcohol but practically insoluble in light petroleum. Ostwald reported that its effects on rape, oats, and peas are similar to those produced by phytohormones—stimulating at low concentrations and inhibiting at high concentrations.

Mention of the Woburn Experimental Station brings to mind the earlier work of Pickering (68-70) who, at the same Station, sought an explanation for the poor condition of young fruit trees when grass was allowed to grow around them. The general result of the presence of the grass was to stop healthy growth, the first symptoms being the light color and unhealthy appearance of the leaves. Although he recognized these symptoms as those of nitrogen deficiency, Pickering was not satisfied that this effect was explained solely by competition, and for many years he sought other reasons. He was a worker of extreme caution, as exemplified by the care with which he examined his criteria of tree vigor. He measured the length of the new shoots and the size of the leaf, and he selected for drying and weighing the sixth leaf from the end of each shoot; he weighed the leaf drop, determined the percentage dry weight and the dry matter of the leaves, their nitrogen content and the total nitrogen of the crop. All these measurements led to the same conclusion as did the weighing of the trees when the experiment was over.

First he dealt with the possibility that the grass robbed the young trees of nutrients. "The grass affects the trees in such a way as to reduce the growth by $\frac{3}{4}$ of the normal amount, even though food material is there, ready for immediate absorption and in quantities 50 per cent greater than in the pots without grass where the trees are flourishing". He concluded that an interference with soil aeration by the grass cover was not the cause of tree injury, for young trees with roots denied access to air by being planted in iron cylinders extending to the clay subsoil, and with the soil covered with two inches of cement made more vigorous growth during the first two years than those planted in open soil and, even after ten years, were better trees than those in unenclosed ground grassed down after planting. That the accumulation of carbon dioxide in soil around the roots of trees in grass was not the cause was shown by the absence of effect of applied carbon dioxide. The soil temperature 6 inches below the surface was lower during the day in tilled soil than in grassed soil but higher at night; yet the difference in summer was on the average only 2-3°F. less than the annual variations of temperature. Did the growth of the grass increase the alkalinity of the soil? Yes, the addition of sodium bicarbonate to the soil slightly reduced tree vigor, but even very heavy doses did not give "an effect at all comparable in magnitude with that produced by grass". Another possible explanation was that the growth of the grass might somehow cause an increase in the fine soil fractions which occur 6-12 inches below the soil and thereby interfere with the action of the tree roots. But sedimentation analyses of tilled, grassed, and alkali-treated soils gave no differences outside the experimental error. A process involving the bacterial numbers of the soil he ruled out by showing that the grass effects appeared even when the trees were grown in sand. The presence of grass on the sand cultures led to an increase in bacterial numbers but the number of bacteria present was below that of the tilled soil.

Pickering was aware of the emphasis placed by the Bureau of Soils on the symptoms of toxin production but was reluctant to accept the idea that roots, as distinct from germinating seeds, could excrete* any solid or liquid "for their function seems to be confined to absorption of such matter as is presented to them in liquid form". Yet, how then could he account for the results of his experiments? In one, for example, iron trays with perforated bottoms were so shaped that they could be fitted around the trunk of apple or pear trees. Grass was grown in sand in the trays which were watered with nutrient solution once a week and with water several times a week in amounts sufficient to keep the pots up to a given weight. The trees with trunks surrounded had a vigor of 68 per cent that of those check trees grown in the absence of grass. It is difficult to avoid the contention that by some means the grass caused the appearance of some substance or other which is actively inimical to the growth of the trees. Yet how such substances arise is a matter of conjecture.

The work of Pickering has been given in some detail for, when reconsidered in the light of these results and those of the later work of Mann and Barnes at Woburn, the broad generalizations of Russell and particularly the earlier conclusion of Clements appear to be weakened. It would seem unnecessary to go to the extreme of Molisch (64) who coined the word "allelopathy" from the reciprocal influence of higher plants. Molisch's experimental work consisted mainly of the growth of plants in an enclosed space in the presence or absence of ripening apples or pears; the effects he observed are attributable to ethylene produced by the ripening fruit. His other demonstrations of the effect of one growing plant upon another were scanty, and scarcely justified the application of so general a term as "allelopathy". Yet the evidence of Pickering and of Mann and Barnes that grasses excrete biologically active substances goes far to justify the conclusion that allelopathy may extend well beyond Molisch's evidence.

What other specific examples are to be found? An early but somewhat mythical example was the rootstock of the tree fern *Cibotium Barometz* J. Smith, which is the plant referred to as the Scythian Lamb by Evelyn in the passage quoted on page 6. In an annotated edition of Evelyn's "*Terra*", published in 1778, A. Hunter added the following footnote:—"This vegetable is called the Tartarian Lamb, from its resemblance in shape to that animal. It has something like four feet, and its body is covered with a kind of down. Travellers report that it will suffer no vegetable to grow within a certain distance of its seat." Hunter referred to Sir Hans Sloan's memoirs on this subject published in the Transactions of the Royal Society, No. 245, p. 461, and mentioned that Mr. Bell in his account of a journey undertaken in 1715 from St. Petersburg to Isfahan reported that the more sensible and experienced among the Tartars treated the whole story as fabulous.

Evelyn also refers to an older observation recorded by Pliny, that "the shade of walnut trees is poison to all plants within its compass," which he refutes by citing the popularity of walnut as a field tree in the wine-growing district of Burgundy.† But incompatibility of black walnut (*Juglans nigra* L.) and butternut (*J. cinerea* L.) in certain plant associations was reported in

*The choice of a word to describe the production of phytotoxic substances from the roots of growing plants seems to have given most writers on the subject some difficulty. Doubtless all the earlier and many recent writers have used such terms as excretion, secretion, exudation, leachate, lysate, diffusate, without attaching any implication of the nature of the physiology of the process. Of these words, exudation is perhaps the most objectionable, for it implies the existence of pores and a secreting mechanism.

†Dr. P. O. Ripley tells me that, although walnut trees are grown extensively in the vineyard areas of Burgundy, the grapes are certainly not grown in their shade.

1925 by Massey (61) and by Schneiderhan (76). Massey suggested that the deleterious effect of the walnut on deep-rooted plants growing in its vicinity is due to juglone, 5-hydroxy-1,4-naphthoquinone. Davis (28) and Gries (37) gave evidence in support of Massey. Gries showed that whereas juglone is present only in trace amounts in the inner root bark of walnut and in the green husk of the fruit, these regions are richer in hydrojuglone, a non-phytotoxic compound which is readily oxidized to juglone on exposure to air. Juglone was found by Gries to be so good a fungicide that he tested the compound as a seed protectant, but it failed because of its phytotoxicity. As a foliage fungicide against black spot of roses, juglone was of promise and caused no damage to the cutinized stem and leaf surface.

A direct ecological significance is granted by Deleuil (29, 30) to toxic root excretions of plants, to account for the absence of annual plants from the heaths of Provence. The plants of these heaths are members of the *Rosemarino-Ericion* association, and Deleuil showed that likely annual seedlings were killed when watered with leachates from these soils. The toxicity ("télétoxie") of leachings from the roots of these plants decreased in the order: *Erica multiflora*, L., *Lithospermum fruticosum*, L., *Helianthemum lavandulaefolium*, Dunal, *Andropogon pubescens*, Willd., etc. Toxicity disappeared when the leachings were heated to 50°C. and was accentuated in soils rich in calcium carbonate. Of the few annuals that survived in these soils, most were either legumes or the semi-parasitic species of *Odontites*. The toxic effects of leachings of the heath soil could be counteracted by watering with macerations of the leguminous root nodules (30).

Since 1950 Guyot and his associates (4-8, 38) at the Ecole Nationale d'Agriculture de Grignon have studied the part played by root excretions in determining the regrowth of vegetation of fallow land or land out of cultivation. In the course of their work they recorded many examples of the inhibitory effect, on the seedling growth of annual plants, of decoctions of the fresh or ground roots of these plants. The water extracts of the roots of mouse-ear (*Hieracium pilosella* L.) or of soil taken from around the roots of this plant reduced the height of seedlings of various crop plants to around 73 and 90 per cent respectively of that of the crop watered with similar extracts of the grass *Brachypodium pinnatum* Beauv. Flax was extremely susceptible to water extracts of the roots of many of the plants tested, from *Hieracium* spp. to the golden rod *Solidago virgaurea* L. Guyot concluded that his experimental data confirm the reality of the root excretions among higher plants: "le concept de la fertilité des terres n'est pas entièrement d'ordre physico-chimique; il est, pour une bonne part, d'ordre essentiellement biologique . . ."

In his account of plant associations in the deserts of Southern California, Went (86) noted that, whereas many desert shrubs such as *Franseria dumosa* Gray were surrounded by many species of annual plants, the shrub *Encelia farinosa* Gray seldom harbored these herbs. Gray and Bonner (35, 36) isolated from *Encelia* leaves a compound, identified as 3-acetyl-6-methoxybenzaldehyde, which was highly toxic to tomato seedlings growing in nutrient solution. As they also showed that the fallen leaves of *Encelia* a year or more old yield toxic rain-water leachings, they suggested that the leaf-produced toxin may be responsible for the absence of annual plants under the bushes of the shrub. Following up this work, Bennett and Bonner (10) examined other desert shrubs for the presence of phytotoxic compounds and found them in *Thamnosma montana* Torr. and Form. and three other species. Three crystalline furocoumarins were isolated from leaves of the former shrub, all phytotoxic to tomato seedlings.

Gray and Bonner's suggestion that the presence of 3-acetyl-6-methoxy-benzaldehyde may have ecological significance was criticized by Muller (65) on the grounds that the leaves of *Franseria dumosa* yield aqueous extracts highly toxic to tomato seedlings growing in water culture. Yet this shrub shelters a rich variety of desert annuals, plants that are not to be found on bare ground except where there is organic debris. Muller considered that the organic debris is necessary for the growing of the annual plants, and that the intricately branched crown of *Franseria* accumulates such debris, whereas none collects around the single root crown of *Encelia*. In partial resolution of this conflict, he suggests that the failure of the toxins to be effective under *Franseria* may be due to absorption by the colloids of the organic debris or to the activity of its microflora.

Bonner's opinion that the toxins eluted from *Encelia* leaves had significance was doubtless influenced by the earlier work of his group on the rubber-producing guayule *Parthenium argentatum* Gray. In the nursery the closely planted seedlings showed so striking an "edge effect" that Bonner and Galston (14, 15) sought the cause. Nutrient solutions leached through guayule plants inhibited the growth of guayule seedlings but had no effect on tomato plants. From the leaching of 20,000 roots of guayule plants they isolated 1.6 g. of a crystalline toxic material which was shown to be *trans*-cinnamic acid. As little as 1 p.p.m. of this compound gave a significant inhibition of guayule plants, whereas tomato seedlings were at least one hundred times less sensitive. The rapid breakdown of cinnamic acid by the soil microflora would reduce any ecological significance the compound may have.

The cinnamic acids have since provided a classical example of the influence of stereoisomers on physiological activity. *Cis*-cinnamic acid was found by Haagen-Smit and Went (39) to have auxin-like properties. Van Overbeek, Blondeau, and Horne (85) demonstrated that the *trans* isomer acts as competitive inhibitor of the natural auxin indoleacetic acid and of other growth-active compounds such as 2,4-D, naphthaleneacetic acid and *cis*-cinnamic acid.

Among other examples of the production of specific compounds toxic to neighboring plants is that of wormwood (*Artemisia absinthium* L.), from the glandular leaf hairs of which Bode (13) isolated a water-soluble derivative inhibitory to the growth of certain weeds. No such inhibiting compound was produced by *A. vulgaris* L., an observation confirmed by Funke (33). Bode concluded that the phytotoxin was a glucoside, but no further work on its nature appears to have been published.

If we turn now to the production of phytotoxins not by living plants but from the decomposing plant material, an early example is provided by Benedict (9). He examined the reasons for the dying out, in spite of good management, of old stands of the brome grass *Bromus inermis* Leyss., and showed that the leachings of dried brome grass roots inhibited the growth of grass seedlings. The nature of the inhibitor is unknown. The suggestion by Stiven (80) that the roots of the grass *Trachypogon plumosus* Nees give rise to a substance that inhibited the growth of weed seedlings was verified by Roux (73), who found an inhibition of germination and a browning of seedling roots of the Kakiebos (marigold) *Tagetes minuta*. The stubble-mulch studies of McCalla and Duley (62) revealed that water extracts of sweet clover hay depressed the germination and growth of maize and, because of the similarity to the effects produced by dilute solutions of coumarin, they regarded the latter compound as a likely cause of the inhibition.

These examples are quoted because the evidence of the presence of a toxic component in the leachings places them apart from the general case of green manuring. The plowing-in of non-leguminous crops, especially if rich in

carbohydrates, may adversely affect subsequent crops by reason of the fact that the increase in numbers of microorganisms engaged in the decomposition of the plant residues leads to depletion of the nitrogen supplies immediately available to the succeeding crop.

The difficulty of establishing young peach trees in old peach orchards, referred to by de Candolle (see p. 7), becomes of practical importance when it is uneconomical to abandon the old site for peach growing, or when the old orchard has to be renovated. The peach replant problem has therefore had special study in southwestern Ontario, reported by Koch (48). The observations of Upshall and Ruhnke (84) that, when an old peach orchard was used as a fruit tree nursery it was possible to tell the site of the peach trees grown six years previously by the poor growth of cherry, pear, and plum tree stocks, indicates that the apparent phytotoxic effect is not confined to peach. Nutritional defects in soil of the former peach tree sites and the presence of greater arsenical residues from sprays applied to the former trees were not demonstrable. Pathological reasons had not been found though, following a report by Johansen (44) that peach replant failures were infested with meadow nematodes (*Pratylenchus* spp.), a prevalence of various eelworm species has been found in old peach soil (48). An earlier suggestion of Proebsting and Gilmore (71) was that one of the causes of tree failure is a phytotoxin produced from root residues of old peach trees. Patrick (61) concluded that this toxic factor is produced by microbial action from amygdalin, a cyanogenetic glucoside present in amounts as high as 5 per cent of the dry weight of peach roots. The enzymic hydrolysis of amygdalin produces hydrogen cyanide and benzaldehyde; the former is known to be phytotoxic but presumably would be rapidly lost by diffusion; the latter would readily be converted to benzoic acid which, like other aromatic carboxylic acids such as the cinnamic acids mentioned above, would be suspected of phytotoxicity.

The phytotoxicity of hydrogen cyanide is considered by Lebeau and Dickson (51) to be responsible for the damage caused by an unidentified fungal pathogen, a low temperature basidiomycete, to alfalfa and other forage plants in the disease known as winter crown rot or snow mold. Snow cover seems to be necessary for the disease to develop and Cormack (26) reported that the extent of damage is related to the speed of thaw, the amount of rot being greater when the snow disappears slowly. Lebeau and Dickson found that the fungus produces hydrogen cyanide when grown on synthetic media, and the amount formed was greater at low temperatures around 4°C., falling off sharply at 16 to 20°C. This example should then perhaps be regarded as one of antibiotic production by fungi, but it is included at this point because of relevance and because hydrogen cyanide production is greater in the presence of alfalfa crop tissue or of soybean meal.

Hydrogen cyanide again appears as the toxic agent in Timonin's work on the varieties of flax resistant and susceptible to the wilt caused by *Fusarium lini* Bolley (82, 83). The nutrient solutions in which the flax plants were grown under aseptic conditions derived hydrogen cyanide from the resistant varieties, whereas susceptible varieties yielded but a trace. Timonin found that the solution from resistant varieties inhibited the growth of *F. culmorum* (W. G. Smith) Sacc. and *Helminthosporium sativum* Pamm. King & Bakke. but stimulated the growth of *Trichoderma viride* (Pers.) Fr. He concluded that the excretion of hydrogen cyanide by wilt-resistant flax influenced the activity of the soil fungi so that the pathogenic forms are suppressed.

Among the parasitic flowering plants the broom rapes (*Orobanchaceae*) are remarkable in that their seeds germinate only when they are adjacent to the roots of their host plants. Lindley (53), in 1846, reported that this condition had been earlier recorded by Vaucher of Geneva. The phenomenon has

been widely investigated since the earlier work of Koch (47) and is of topical interest because of the recent discovery of maize in North Carolina parasitized by a species of *Striga*. Following up the observations of Saunders (75), of Barcinsky (2), and of Chabrolin (24) that the seeds of *Striga lutea* Lour., of *Orobanche cumana* Wallr. and of *O. speciosa* respectively would germinate only if stimulated by leachings of the roots of their host plants, a group of workers of Leeds and Cambridge Universities (16-20) have examined the effects of the percolates from growing sorghum, linseed, and other plants on the germination of *S. hermontica* Benth. and of *O. minor* Sm. It was found that the activity factor could be removed from the percolates by adsorption on activated charcoal from which the compounds responsible could be eluted with aqueous acetone.

By this treatment of leachings from seedlings of *Sorghum vulgare* Pers., Brown, Johnson, Robinson, and Todd (19) found that the stimulant to *Striga* seeds contained carbohydrates including free pentoses. Tests of a number of the sugars showed that D-xyloketose was a powerful stimulant, active at concentrations of 1×10^{-6} mg./ml. Chromatographic studies (20), however, showed that the germination factor may be complex and that its most effective component is neither a simple sugar nor an amino acid.

A chromatographic study (18) of the factors recovered from leachings of linseed which stimulate the growth of seeds of *O. minor* led to the conclusion that the active compounds were akin to those derived from sorghum, though none of the sugars tested were found to be active. Purification by counter-current extraction (17) failed to yield the factor in a pure state but provided information on its chemical properties. The factor approximates in ultimate analysis to $C_{11}H_{16}O_4$; its infrared spectrum indicates the presence of a hydroxyl group, two ester or δ -lactone groups, a methyl group and a double bond. The similarity of physical and chemical properties suggests that the compounds active on *Striga* and on *Orobanche* have features in common, and indeed that such properties are shared by the factors associated with the stimulating effect of the host plant root extracts that encourage the emergence of the larva of the potato eelworm from its cysts.

The cyst-forming nematodes of the genus *Heterodera* are remarkable in that the female after fertilization retains the eggs within her body, which degenerates to form a cyst highly resistant to drought and to the attack of other soil organisms. The host plant range of the various species of *Heterodera* is generally limited, and in the more highly specialized forms the larvae will emerge from the cysts only when these are stimulated by the root diffusates of the host plant. This phenomenon was first observed by Baunacke (3) in both the beet eelworm *H. schachtii* Schmidt and the potato eelworm *H. rostochiensis* Wollenw. The stimulant effective on the latter is present in leachings from growing solanaceous plants, and in the absence of the stimulant the cysts remain dormant. Cysts of the cabbage eelworm *H. cruciferae* Franklin were found by Winslow (91) to hatch freely in leachings from hosts in the genus *Brassica* but not in those of other cruciferous plants, though the latter caused high larval emergence from the cysts of the beet eelworm.

This selectivity of action is powerful evidence of the excretion from the growing host plant of a chemical or chemicals each specifically active in stimulating emergence from cysts of the appropriate species of eelworm.

The economic importance of the cyst-forming eelworms and the difficulty of their control by chemical or cultural methods has enhanced interest in the problem of elucidating the nature of those stimulatory excretions. Despite prolonged research at Cambridge and at the Cornell Agricultural Experiment Station, the responsible chemicals have not yet been identified. The material from the leachings of growing tomato plants and effective on cysts of the

potato root eelworm, recovered by chromatographic methods and by fractionation of the brucine salt, was named eclipic acid by the Cambridge workers (21, 22, 60). On the assumption that the acid isolated by these workers contains but minor amounts of impurities, it was deduced that it had a molecular weight of about 300, an equivalent of 250—290, and a γ -lactone group of which the hydroxyl group is not tertiary. On this and other evidence it was inferred that eclipic acid is of formula $C_{19}H_{26}O_8$, which is in reasonable accord with the molecular weight and will accommodate the anticipated carboxyl, lactone, and two hydroxy groups of which one has acidic properties. Parallel tests of the activity of compounds that satisfy these requirements revealed that anhydrotetrone acid stimulated emergence from cysts and that the larvae that emerge behave as do those released by eclipic acid. Although the concentrations required of anhydrotetrone acid were above those required of the more potent samples of eclipic acid, the former compound has been used for the artificial stimulation of larval emergence by Bishop (12).

A more subtle method by which the stimulatory root excretion may be utilized for the practical control of eelworms was tried by Hijner (43). He showed that root diffusates of the wild beets *Beta patellaris* Moq., *B. webbiana* Moq. and *B. procumbens* Lange were as active as those of sugar beet in stimulating emergence for cysts of the beet eelworm *H. schachtii*, but that the larvae were incapable of reaching maturity in these hosts. The planting of *B. patellaris* on heavily infested soil in May reduced in five months by about 90 per cent the content of viable cysts in that soil. Jones (45) had previously shown that *H. schachtii* was incapable of developing in swine cress *Coronopus squamatus* (Forsch.) Aschers, and Winslow (91) found that leachings from swine cress effectively induced emergence from cysts of *H. schachtii*. Jones (46) reported that lines of *Solanum andigenum* resistant to the potato root eelworm all produced a root diffusate but slightly less active than that of susceptible potato varieties. Resistance, therefore, appears to be associated with the poor development of the larvae on the resistant host. Whether or not their failure to develop is due to malnutrition or to a toxic agent is beside the point, for there is no line of division between malnutrition (as distinct from starvation) and toxication.

This evidence of the existence, in root diffusates, of highly potent and selective materials effective, in the latter case, in stimulating emergence from eelworm cysts and, in the former case, in permitting germination of seeds of parasitic flowering plants, encourages a more extensive search of the literature on the factors influencing seed germination.

As a general case, the seed of plants although capable of germination do not germinate while still within the fruit or pod, except when it is exposed to unnatural conditions—as in the refrigerator. One explanation of this is the presence of an inhibitor in the fruit. Koeckemann (49) postulated the presence, in the pulp of apples, of germination inhibitors to which he gave the general name "Blastocholines". Though Tetjurev (81) concluded that the blastocholines were merely fruit acids such as maleic and citric acids, the term has survived. Moewus, Moewus, and Schader (63) examined the germination and seedling growth of cress and wheat, and demonstrated that blastocholine was present in thirty-three genera of fruits from sixteen widely spread orders of plants. Nor are such inhibitors confined to the fleshy fruits—Evenari (32) gave an extensive list of plants capable of producing germination inhibitors in seed coat, in leaves, and in other plant parts.

A remarkable example is derived from Went's work (87, 88) on the biotic factors influencing the germination of seeds of desert plants in the deserts of Southern California. He traced three causal mechanisms: firstly, the mechanical abrasion by silt and sand in the runoff after heavy rain; secondly,

the leaching from soil of water-soluble salts by heavy rains (for the seeds of many of the desert varieties would not germinate in even a slight concentration of inorganic salts in the soil); thirdly, the removal of water-soluble inhibitors from the seed by the action of the rain (for if the leached seeds are placed in their own diffusates germination is inhibited).

It is apparent that the inhibitory mechanism, whereby the germination of the seed is delayed until the environment is favorable, has additional phytosociological significance if the inhibitory compound is effective against the seeds of other plant species. Evenari (32) reviewed the chemical data of the blastocholines identified before 1949, and prominent on the list are the organic acids of fruit juices, and the glycosides and their hydrolytic products, for example, the cyanogenetic glycosides of the *Prunacea* and *Pomaceae*, and the mustard oils of the cruciferous seeds. In his examination of the root excretions of oats by fluorescent and ultraviolet light, Eberhardt (31) established the presence of two glycosides, one of which he identified as the glycoside of scopoletin; scopoletin and an unidentified non-glycoside were also present. The pronounced activity of scopoletin and other coumarin derivatives in plant growth is well established (see 34) by way, as Andreae (1) showed, of their effects on the reactions of the phytohormone indoleacetic acid. Eberhardt considered, however, that as the fluorescent coumarin derivatives which he found to be excreted from oat roots are decomposed rapidly in natural soil, their ecological significance is not important.

The inhibitory compounds listed by Evenari are generally phytotoxic, but many examples of specific activity are to be found—a feature that adds scientific interest to their study. Went, Juhren, and Juhren (89) found that leachings from growing barley inhibited the germination of seeds of *Amaranthus hybridus* L. and *Chenopodium album* L., both known by the popular name of pigweed, whereas leachings from growing tobacco plants inhibited seeds of *A. hybridus* but not of *C. album*.

It would be misleading to conclude this chapter without further reference to the importance of the actions of the natural auxin, 3-indoleacetic acid, on the germination and growth of plants. These reactions provide ample scope for the explanation, not only of the dormancy of the seed or bud, but of the action of natural inhibitors. The suppression of the development of lateral buds during the growth of the apical buds of shoots is now thought to be an inhibition by the auxin coming from the terminal bud. The sprouting of shoots of potato tubers at the close of the dormant period, or when the tubers are treated with dormancy-breaking chemicals such as ethylene chlorohydrin, was shown by Hemberg (41, 42) to be due to the disappearance of large amounts of growth-inhibitory substances that had been in the peel during dormancy. The nature of their inhibition and its relationship to the content of free and bound auxin has not yet been elucidated. Bentley and Bickle (11) adduced evidence that the neutral growth inhibitor isolated by Stewart (79) may be 3-indolylacetonitrile.

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Chapter 3

HIGHER PLANTS *vs.* INSECTS:

Being Mainly Concerned with the Chemical Defenses of Plants against Insects

Of the herbivorous animals, the phytophagous insects and mites exhibit in the most marked degree the phenomenon of host selection. Among higher animals examples such as the koala *Phascolarctus cinereus*, with its diet limited to eucalyptus leaves, are rare. Among insects it is a frequent rule that each species is restricted to but a few species of the plants of its environment; indeed, in the case of many insects, insect damage to a particular crop may be reduced by the growing of varieties of that crop plant that the insect is unable or unwilling to attack.

The development and use of insect-resistant varieties of crop plants was reviewed in a thorough manner by R. H. Painter in his book "Insect Resistance in Crop Plants" published in 1951. It is therefore unnecessary here to cite successful examples of that practice except when the cause of resistance is thought to be chemical. On the other hand the examples cited below have been gleaned, not only from crop husbandry, but also from the broader fields of chemotropism if they relate to possible mechanisms involved in host selection by phytophagous insects.

Host selection, if it implies that the insect is capable of a purposeful choice, is an unfortunate name to describe the restriction to but a few host plants of the phytophagous insect. In general, no choice is involved. With the peach aphid *Myzus persicae* Sulz, which thrives in its viviparous stages on many species of host plant, the ovipara can live only on the peach and a few other *Prunus* spp. Broadbent (4) concluded that the alatae of this aphid find the peach tree only by chance but, having found it, remain on it crawling until they find a suitable leaf. If a choice is made it is in most cases by the gravid female parent, for the feeble larva newly emerged from the egg must either starve or feed on the host tissue that happens to be near its mouth parts. The egg-laying female is, on the other hand, equipped with powers of locomotion and with highly developed sense organs.

The so-called Hopkins Host Selection Principle* (27) which, to quote from Dethier (12), "states in essence that an oligophagous or polyphagous insect prefers to oviposit on the food upon which it fed as a larva" provides no mechanism whereby this "larval memory" can become operative, but the most plausible hypothesis is of an olfactory response. Certainly there are abundant examples of the extreme sensitivity of many species of male moths to the attractive chemicals arising from female moths (see p. 67). Moreover, there is ample proof that chemotropism is involved among the hymenoptera parasitic

*Although this principle has been named after A. D. Hopkins, Craighead (7) pointed out that the same idea, though with Lamarckian implications, had been put forward as early as 1864 by Walsh (59) in the following terms: "We might infer *a priori* that when from peculiar circumstances a Phytophagic Variety, including both the sexes, has fed for a great many generations upon one particular plant of the number inhabited by the species to which it belongs, it would be likely to transmit to its descendants in the imago state a tendency to select that particular plant upon which to deposit its eggs."

on other insects, among insects infesting man and stock, and among saprophagous insects whose larvae feed on decaying or fermenting organic matter. Examples from these groups have already been reviewed by Dethier (12).

The factors determining the choice of host plant for oviposition by phytophagous insects are less clearly known. McColloch (44) concluded that the odor of the silk of Indian corn may be of importance in attracting adults of the corn earworm (*Chloridea obsoleta* F.), and he secured egg-laying on twine soaked with fresh corn silk juice. Lipp (40), noting the presence of garlic in turf destroyed by the Japanese beetle (*Popillia japonica* Newm.), exposed dishes containing dilute alcoholic solutions of allyl sulphide, or of alcohol alone, and found that the beetle laid numbers of eggs in the sod adjacent to the allyl sulphide dishes.

Although olfaction may be the mechanism by which the female is attracted to her host plant, other responses may determine the site of oviposition. Thus Crombie (8) obtained olfactory responses of the lesser grain borer (*Rhyzopertha dominica* F.) to maize and cereals, and considered that the sense of touch was used to find a suitable place to lay the eggs. It may be noted that Crombie could detect no preference by the beetles for oviposition or feeding on the food on which they were fed as larvae. No other record has been found of the experimental study of the Hopkins Host Selection Principle—perhaps because of difficulties associated with the rearing, to egg-laying stages, of larva fed on a plant not its natural host.

A number of shorter-term experiments are reviewed by Dethier and Chadwick (15), and the general conclusion is that, if it is possible for insects to become accustomed to abnormal food, this "conditioning" is lost within a few hours. The term "conditioning" may be most easily explained by an example. Thorpe and Jones (55) showed that the ichneumonid *Nemeritis canescens* Grav. normally is parasitic on larvae of the meal moth *Ephestia kuehniella* Zell., which it finds by means of a chemical stimulus detected by receptors on its antennae. If larvae of the small wax moth (*Achroia grisella* F.) are contaminated with the smell of *Ephestia*, the parasite will oviposit on this new host and will develop normally. *Nemeritis* reared on *Ephestia* showed no reaction to *Achroia*, but those reared on *Achroia*, when given a choice of the two hosts, showed a conditioned attraction to *Achroia* which reduced from 85 per cent to 65.8 per cent the number choosing *Ephestia*. A recent example is given by Montieth (47) reporting on *Drino bohémica* Mesn., a dipterous parasite of several species of sawflies each preferring different coniferous host plants. He showed that the choice of the parasite was influenced, not only by olfactory responses to the foliage upon which the host was feeding and to the host, but also by chemotactile responses to the host itself. Continuous breeding of the parasites in different sawfly species led to changes in the order of preference of the offspring when compared with the preference of the parents; but such changes were not cumulative and did not persist. Those sawfly hosts selected most frequently by the parental parasites were also selected most frequently by the offspring, nor was any preference developed for the food plant of the host on which the parasites developed. Montieth concluded that "conditioning" is of little importance as a reason for the evolution of the biological races of *D. bohémica* which parasitize different host sawflies.

On the other hand, much is known of the factors determining the range of plants acceptable as food by phytophagous insects. Clearly, we are not here concerned with the coincidence of distribution of host plant and pest as determined by soil and climatic conditions; our purpose is the discussion of the chemical factors that determine whether or not the plant will serve as a host for the insect.

Dethier (13) found it convenient to differentiate three stages in the process by which the insect infests its host plant: (i) the finding of the plant, (ii) the initiation of biting, and (iii) continued feeding. If the feeding stage of the insect pest has not already been brought to its host plant by ovipositional stimuli, it may reach the host through reason of vision, light, gravity, or moisture; but chemotropism is probably the important cause. That this is the case for foraging insects such as bees was shown by the early work of von Frisch (24), who trained honey bees to select, from other sugar solutions, the solution flavored with an essential oil. The olfactometer studies of McIndoo (45) and of Folsom (22) showed that the Mexican boll weevil *Anthonomus grandis* Boh. is attracted to the cotton plant by odors, which Folsom ascribed to ammonia and to trimethylamine.

It is not always easy, in the interpretation of early work on this subject, to distinguish between the factors attracting the insect to its host and those responsible for its feeding—in anthropomorphic terms, to distinguish smell and taste. When a reaction is obtained to a non-volatile compound, however, it could hardly be regarded as olfactory and must presumably be a gustatory response.

Advantage has been taken, in studies of the gustatory responses of insects, of the extension of the proboscis with which many butterflies and flies respond to the contact of their tarsi with sugar solutions. By using this reaction of blowflies *Phormia regina* Meigen whose antennae and labella had been removed to eliminate response to odor, Chadwick and Dethier (5, 6, 14, 16) were able to trace with accuracy the relationship of molecular structure and chemoreception of homologous series of alcohols and glycols, of aliphatic acids, aldehydes, and ketones.

The first recorded attempts to induce insects to feed on plants other than their known hosts are those of Grevillius (25), who showed that larvae of the brown tail moth *Euproctis chrysorrhæa* L. taken from chickweed would feed on other plants, if the latter were smeared with a paste prepared from chickweed. Verschaffelt in 1910 (58) observed that the larvae of the cabbage butterflies *Pieris brassicae* L. and *P. rapae* L. fed only on plants containing mustard oil glycosides. He induced the larvae to feed on other plants by treating their leaves with preparations containing the glycosides or the mustard oils. Thorsteinson (57) extended Verschaffelt's line of attack to the larvae of the diamondback moth (*Plutella maculipennis* Curt.) and showed that the addition of sinigrin, sinalbin or glucocheirolin made pea leaf powder an attractive food to these caterpillars. The gustatory stimulus was reduced when the glycosides were first subjected to enzymic hydrolysis, an indication that the mustard oils themselves do not induce prolonged feeding, even though they produce a marked olfactory response.

Another example of differentiation between olfactory and gustatory stimulation appears in the feeding habits of wireworms (*Agriotes* spp.). Falconer (20) concluded that wireworms have no means of locating their food. Thorpe *et al.* (56) could not obtain responses of wireworms in the olfactometer, but found that contact with various carbohydrates, triolein, or animal proteins elicited a biting response. The larva was particularly sensitive to asparagine and related amines. Thorpe and his colleagues suggested that such protein-degradation products set free in the soil by the roots would keep the wireworms in the vicinity of the roots and thereby provide a food-finding process. This suggestion is an adequate explanation of the successful use of trap crops for wireworm control.

The converse of attractivity is repellency and, though in theory, it is possible to distinguish between a true repellent and one that masks an attractive odor, no clear-cut difference has been found in practice. Nor can

examples be found of ovipositing insects being dissuaded from depositing their eggs by host plant odors, though there are examples among insects parasitic on other insect species of a chemotropic avoidance of oviposition. Thus Salt (53) showed that females of *Trichogramma evanescens* Westw. are deterred from oviposition on a parasitized host by volatile substances secreted by the original egg-laying female. This and other cases led Flanders (21) to consider odor of paramount importance in the stimulation or repression of reproduction among parasitic insects. Moreover, there are several instances of high practical significance of compounds repellent to mosquitoes and biting flies though, with the exception of oil of citronella and related terpenes, the repellents used for the protection of man and stock are synthetic compounds, such as dimethyl phthalate and 2-ethyl-1,3-hexanediol, suitable for application to human skin, butoxy-polypropylene glycol for protection of dairy cattle, and *N.N.*-diethyl toluamide for the treatment of clothing.

From the agricultural point of view the examples of deterrent action as a reason for the avoidance by insects of certain possible host plants are of great significance, for many of the known cases of crop varieties resistant to insect attack are due to these reactions. Many such cases can be attributed to physical factors that render the resistant variety an unsuitable host, features such as the longer hairs of the varieties of cotton that are resistant to the jassid *Empoasca facialis* Jac. Parnell, King, and Ruston (49) established a close and consistent correlation between the degree of hairiness of the under-surface of the cotton leaf and the degree of resistance to the jassid. Not so, however, with the related jassid *E. devastans* Dist., for Husain and Lal (28) found that not all hairy Indian varieties of cotton were resistant to this insect. Most of the imported American cotton varieties were susceptible, whereas native Indian varieties had a degree of resistance, which seemed to arise through a reduced response of the plant to the phytotoxic insect saliva.

In some Balkan countries another mechanical example is found in the old practice of ridding an infested house of bedbugs by strewing leaves of French bean *Phaseolus vulgaris* L. on the floor (51). The leaves of this bean variety have hooked epidermal hairs, which entrap not only the bedbug but other pests such as the aphid *Myzus persicae* (46) and *Aphis craccivora* Koch (30).

The control of insect pests by the use of resistant varieties of crop plants is so attractive and economical a method of crop protection that it has received wide study. Whether or not it would be feasible, in cases where resistance is due to the presence of toxic or deterrent substances, to apply such substances to the protection of susceptible varieties is a question that has not yet been put to practical test; but the ecological significance of such substances is obvious. In some cases, the substance might act in a mechanical fashion as, for example, the gummy material exuded from tomato leaves that protects the tomato from infestation by *M. persicae* (46). Leaves of *Solanum polyadenium* exude a substance that so impedes the movement of aphids that this plant is resistant to aphid attack (54). This species of *Solanum* has been used as genic material in the breeding of potato varieties that, by being resistant to aphids, become less liable to infestation by aphid-borne virus diseases.

Similarly, there are *Solanum* species resistant to the Colorado potato beetle *Leptinotarsa decemlineata* Say.; and the work of incorporating the genes causing this resistance into the potato is in active progress in many European countries invaded by the beetle in the past decade. Kuhn and Löw (33) have given an account of the work that they and their colleagues have carried out at Heidelberg on the nature of this resistance. The leaves of *Solanum demissum* are eaten by beetles but not by larvae; the leaves of *S. chacoense* are refused by both beetle and larva. This difference was found to be due, in part at least, to alkaloidal glycosides present in the leaves. The deterrent nature of

these alkaloids was established, after their isolation and crystallization, by spraying the pure alkaloid in gelatin solution on the potato leaves and observing the reactions of the beetles or larvae placed on the sprayed leaves. The favored method, however, was by infiltration: the leaves of the potato plant were placed in solutions of glycoside to be tested, and the whole was evacuated to remove air from the leaves; when the vacuum was broken the solution was taken up by the leaves in amounts that could be weighed. The treated leaves were then offered as sole source of food to larva or beetle, and the fate of the insect was determined after a period of ten days.

The only alkaloidal glycoside previously found in the leaves of *S. tuberosum* L. is solanine, first discovered in 1822 in the leaves of *S. nigrum* L. and *S. dulcamara* L. On hydrolysis this glycoside yields solanidine, the structure of which is given on p. 35. Kuhn and his associates isolated six glycosides from *S. tuberosum*. These glycosides include three solanines: α -solanine, which appears to be identical to the previously-described solanine, is a trisaccharide, the sugars being galactose attached to the hydroxyl of carbon 3 of solanidine, then glucose and then rhamnose; β -solanine, which lacks rhamnose; and γ -solanine, which, lacking both rhamnose and glucose, is the galactoside of solanidine. The β - and γ -solanines either may be intermediates in the bio-synthesis of α -solanine or may arise by metabolic breakdown or as artefacts in the isolation of the alkaloids. In addition to these three solanines the Heidelberg workers isolated from *S. tuberosum* small amounts of three other glycosides, which they found to constitute almost half the glycoside content of *S. chacoense*. They were unable to isolate the aglycone of α -chaconine, for it appears to lose readily one mole of water giving solanidine, which is the aglycone of both β - and γ -chaconines: the three chaconines followed the pattern of the solanines, giving on hydrolysis:

α -chaconine: solanidine + glucose + rhamnose + rhamnose;

β -chaconine: solanidine + glucose + rhamnose;

γ -chaconine: solanidine + glucose.

The larvae are unaffected by amounts of α -solanine, α - and β -chaconine well above those present in potato leaves; hence the cause of the resistance of *S. chacoense* is still unknown.

The main alkaloidal glycoside recovered by Kuhn and his colleagues from leaves of *S. demissum*, the leaves of which are eaten by the larvae but not by the beetles of *L. decemlineata*, was named demissine. On hydrolysis it proved to be the tetrasaccharide. From preliminary chromatographic studies they concluded that the sugar component is probably identical to that of tomatin (an antibiotic first isolated from *Lycopersicum pimpinellifolium* Dunal, and which is described on p. 34), for it is toxic to certain fungi and bacteria. Tomatin has since been isolated from several species of *Lycopersicum*, including the tomato, and was found by Kuhn *et al.* to be active against the larva of the Colorado beetle. The resistance of *S. demissum* to the larva is therefore ascribed to the content (up to 0.5 per cent) of tomatin.

The relative freedom of soybean plant from insect infestation, at least in the United States of America, and of soybean products from attack by stored insect pests, was attributed by Lipke, Fraenkel, and Liener (39) to the presence of a heat-labile substance, probably protein in character, that interferes with digestion of protein by the insect. This antiproteolytic toxin has not been further characterized, but it is distinct from soyin (38), which is held responsible for the poor nutritive value to vertebrates of untreated soybean protein.

Although, in these examples, the repellent compounds present in the tissue of the resistant host are not only deterrent but are actually toxic to the insect, it does not follow that the presence of an insecticidal compound will

confer insect resistance to the host plant. Nicotine for example is a potent aphicide, yet the presence of nicotine and allied alkaloids in tobacco does not seem to protect that plant from aphids and white fly, insects that may easily be killed by the application of nicotine, either in a well-wetting spray or as a fumigant. The suggestion that the insect fails to ingest nicotine from the tobacco leaves by an artful disposition of its stylet seems an unsatisfactory explanation, for the insects consume large amounts of sap, and it seems likely that the sap would contain the alkaloids. The grafting experiments of Dawson (11) and of Hieke (26) reveal that, when tomato is grafted on stocks of *Nicotiana tabacum* L., the tomato leaves accumulate nicotine. The simplest explanation of these grafting results is that the biosynthesis of nicotine occurs in the tobacco roots, from which it is translocated to the leaves and should therefore reach the sap-feeding insect. But the results of other grafting experiments to determine the site of biosynthesis of the alkaloids do not always support this simple hypothesis. As this subject has been recently reviewed by James (29) it need not be pursued here.

It may be argued that nutritional factors alone suffice to explain host plant specificity. This topic was well discussed in a symposium at the 9th International Congress of Entomology and there Fraenkel (23) reviewed the nutritional value of green plants for insects. He concluded that, as green leaves are excellent sources of all the food materials that insects appear to require, host plant specificity is determined by the presence or absence of odd chemical substances such as glucosides, essential oils, alkaloids, and tannins. He admitted that this was an extreme view, and pointed out that little is known of the food requisites of phytophagous insects or of the effect of chemical changes in the composition of the leaves on the development of insects.

In the studies of Auclair and Maltais, nutritional factors seem to provide an adequate explanation of the degree of attack on different pea varieties by the aphid *Acyrtosiphon pisum* Harris. Maltais (41) established a correlation between the total water-soluble- and amino-nitrogen of the tissue of pea varieties and their susceptibility to attack by this aphid. Auclair (2) narrowed the basis for this correlation: he found that the amino acids arginine, threonine and valine, known to be essential for growth, are present in much higher amounts in susceptible than in resistant varieties—non-essential amino acids, such as alanine, glutamic acid and proline, being present in about equal amounts in resistant and susceptible pea varieties.

On account of their life history, many aphid species make suitable test animals for the study of the effects of nutrition and environmental factors on behavior and fecundity. The apterous alienicolae reproduce so rapidly that they are mainly responsible for the summer infestation of the host plant. Davidson (9) and Lathrop (34) found that, in this stage, the length of time between birth and the production of the first brood varied inversely as the temperature. Evans (19) found that, on plants grown under normal conditions of sunlight, the rate of reproduction in the cabbage aphid *Brevicoryne brassicae* L. was controlled by the nitrogen content of the host plant.

Sooner or later winged forms appear among the alienicolae, and it has been suggested that the production of winged forms is due to overcrowding and starvation (1), or to overcrowding even in the presence of abundant food (50). Rivnay (52) did not find that overcrowding caused winged development but considered that loss of water, rather than the amount of nutrient, effected the appearance of winged forms, which followed a reduced water intake or an exposure to a low atmospheric humidity, or when the aphids were feeding on the older mature leaves—which presumably had a lower water percentage than the younger and more succulent growing tips. Evans (19) showed that

the proportion of winged forms of *B. brassicae* was greatly increased as the protein content of the host plant fell below 0.2 per cent protein nitrogen, though wide variation in the carbohydrate content of the plant had no effect on the production of winged forms. It would appear, from the diversity of the results in this limited evidence, that an explanation of the influence of environmental and host plant factors on the life history of the insect, on a nutritional basis, is not going to be simple.

An earlier hypothesis, that day length is responsible, was advanced by Marcovitch (42, 43) on the evidence that the males and sexuparae of three aphid species appeared earlier in the growing season of their hosts if the latter were exposed to short days for about seven weeks. Conversely, he found that the appearance of migratory forms of *A. sorbi* Kalt. (= *Anuraphis roseus* Baker) on its winter host was associated with the increased day length of the spring months. This hypothesis is supported by the observations of Davidson (10) on *Aphis rumicis* L. and of Wilson (61). The latter worker studied *A. chloris* Koch, whose host range is apparently restricted to the genus *Hypericum* and whose life history seems to be governed solely by the environmental factors of temperature and day length. Given high temperatures and many hours exposure to light the aphid remained parthenogenetic; low temperatures and short exposures to light caused the appearance of oviparous forms. Kenten (31) obtained like results in her studies on the pea aphid *Acyrtosiphon pisum*, for sexual forms were produced at temperatures below 20°C. only by those parent aphids that had received a photoperiod of 8 hr./24 hr. during their nymphal period; parents, held at 25-26° and at 29-30°C., and receiving a photoperiod of 16 hr./24 hr. during their nymphal period, produced no sexual forms.

The mechanism of this phenomenon is unknown, but Davidson (10) assumed that the initial effect of photoperiod is on the host plant: day length, by affecting photosynthesis, would affect the carbohydrate content of the sap, and thereby affect the nutrition of the aphid. Since Davidson's time, however, the significance of day length or photoperiod on the physiological processes of plants, and particularly of flowering, has been exposed. It is now generally accepted that photoperiodism is a hormonal phenomenon, whether due to a specific chemical such as the hypothetical "florigen" or to a specific inhibition affecting the auxin mechanism. It would accordingly be simple to suppose that the effect of photoperiod on the aphid's life history is likely to be associated with a phytohormone, but for evidence derived from studies of diapause in certain leaf-eating insects.

Diapause in insects is a stage of arrested development which, in some species, can be brought about by exposure to adverse environmental conditions, but which cannot be broken merely by an improvement in these conditions. In other species it will occur even under favorable environmental conditions—a fact which adds difficulty to the use of such insects as experimental animals. Dickson and Sanders (17, 18) met these difficulties in rearing the Oriental fruit moth (*Grapholitha molesta* Busck.) and found that, by exposing the apples on which the larvae were feeding to long days of 15 hours or more per day, the number of larvae entering diapause was reduced from about 98 per cent to 5 per cent or less, a rise of temperature from 70° to 80°F. having a much smaller effect. Way and Hopkins (60) obtained a similar striking effect on the number of pupae of the tomato moth *Diataraxia oleracea* L. entering diapause when the larvae and the leaves on which they were feeding were exposed to day lengths greater than 15 hours. The light intensity, if above a certain minimum, had no influence—though, when the larvae were reared in darkness, diapause was prevented by high temperatures (30-34°C.). Way and Hopkins tested whether or not the effect was produced by a photo-

periodic response of the host plant by feeding the larvae under long photoperiod on leaves, changed twice each day, from plants growing under short photoperiod. As the resulting pupae did not enter diapause, they concluded that the photoperiod effect was exerted directly on the larvae. The work of Lees (35, 36) on the phytophagous mite *Metatetranychus ulmi* Koch led to a similar conclusion. This mite develops from two types of egg: "summer" eggs laid on the leaves of the host plant, which develop without interruption; "winter" eggs laid on the bark, which hatch only after an intervening period of diapause. Lees found that whether the female lays eggs of the "summer" type or of the "winter" type is determined by nutrition, photoperiod, and temperature. If, for instance, the leaves of the host plant are so heavily infested that they "bronze", Kuenen (32) observed that the mites lay "winter" eggs at an early date. With days 0-13 hours long and temperatures at about 15°C., Lees found only "winter" eggs were laid, though high temperatures of 25°C. tend to eliminate diapause even with short photoperiod. At continuous illumination only "summer" eggs are laid. The recurrent transferring of mites showed that on long-day host plants mites living under short-day conditions gave "winter" eggs, whereas on short-day host plants mites living under long-day conditions laid "summer" eggs. Lees therefore concluded that the mites are influenced directly by day length, and not indirectly by the effect of photoperiod on the host plant.

A precedent for belief that the photoperiod effect on insects is direct may be found in the experiments of Baker (3) with larvae of seven species of mosquito. He concluded that the shortening day is the dominant factor in initiating a rest period in the autumn, and the lengthening day in bringing about larval activity in the spring.

The remarkable effect of day length on the activity of migratory birds and poultry is now well established. Indeed, Parker and his colleagues (48) have suggested that the photoreaction that controls flowering in plants is the same as that which controls the plumage cycle of birds, the production of wings by aphids and the prevention of diapause in some insects, and which regulates the hair cycles of certain animals. In all of these reactions the effective wavelengths (around 6250Å) are similar; and the evidence suggests that the controlling process involves slow reactions occurring in the dark period. Lees (37) pointed out, however, that the photoperiodic mechanism in arthropods differs fundamentally from that of plants in that with arthropods, slow timing reactions take place during both the dark and light periods. It would therefore appear that photoperiodism in phytophagous insects is not dependent on the formation of a physiological active chemical in the host plant.

The hormonal control of diapause and of metamorphosis in insects is now established; as the existence of the active chemicals has been demonstrated, it should not be long before their isolation and identification is accomplished. The mechanism whereby photoperiod affects the production and reactions of these hormones may then become apparent.

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Chapter 4

HIGHER PLANTS vs. FUNGI:

The Chemical Defenses of Plants Against Fungal Attack

In general, it may be said that there is no evidence that the normal plants are able to attract or repel pathogenic or mycorrhizal fungi, though an exception may emerge in the immediate neighborhood of the plant roots. This exception is discussed further in the succeeding section dealing with the rhizosphere. Moreover, there is little evidence that the host plant has any influence either on the germination of the fungus spore or of the growth of the fungus prior to the establishment of infection except by the plant's non-specific metabolic products—carbon dioxide (5) or nutrients exosmosed into moisture on the leaf surface (1).

Gäumann (9) has classified the various mechanisms by which higher plants may prevent invasion by pathogenic fungi, and may resist or limit attack by fungi—whether pathogenic or mycorrhizal. Broadly speaking, the most successful of the plant defenses is the rapid death of the tissues adjacent to the site of the infection, whereby those pathogenic fungi which are so specialized that they cannot survive saprophytically are deprived of nutrient. The death of the adjacent plant tissue is presumably due to the toxins produced and, as it seems reasonable to assume that the associated chemical is a fungal rather than a plant product, a discussion of the nature of these toxins is reserved for the chapter on the interactions of fungi and higher plants (pp. 43-52).

A second type of mechanism, the formation of a protective barrier, whether it be suberized tissue or gum, may more logically be regarded as a reaction of the plant itself. Gum formation is, in those plants capable of it, a general reaction to tissue damage and is to a large extent independent of the cause of injury. It is perhaps significant that many plant gums and resins contain terpenes or aromatic derivatives of marked fungicidal and bacteriocidal properties.

The colorimetric reactions of hydroxyaromatic compounds such as the phenols permit their ready detection by histochemical techniques. The frequent presence of such compounds in plant tissue prompted the suggestion that they function as anti-fungal compounds. Dufrénoy and his colleagues (3, 4) considered that the accumulation of such phenolic compounds in plant cells adjacent to those invaded by pathogenic fungi is evidence of a defense mechanism. Newton and Anderson (18) attributed the resistance of certain wheat varieties to rust to the presence of phenols; Gäumann (9) was skeptical, for these phenols occur in the tissues of susceptible as well as of resistant varieties.

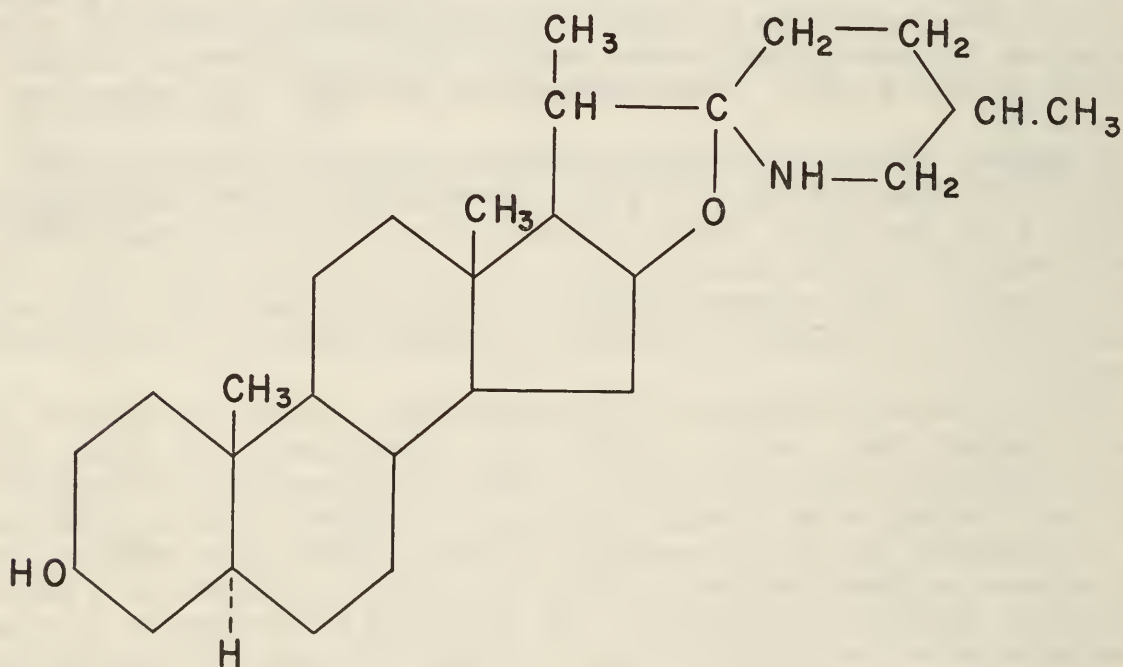
A clear-cut example is due to Walker and his associates (11, 19), who showed that the resistance of onions to *Colletotrichum circinans* (Berk.) Vogl. is a genetic factor linked to scale color and that the resistant principle resides in the phenolic substances, mainly protocatechuic acid and catechol, associated with pigment metabolism. Because the resistant variety is attacked by the fungus if the dried outer scales of the onion are removed, it would appear that the inhibitory phenols are either present in the living tissue in some non-toxic

form or do not come into contact with the invading pathogen. When, however, the outer scales become desiccated the toxic phenols are liberated or become available for the protection of the living scales of the resistant onion.

The resistance of rye to *Fusarium nivale* (Fr.) Ces. was traced by Virtanen and Hietala (23) to the production of 2(3)-benzoxazolinone, which they recovered in amounts up to 0.01 per cent from fresh rye seedlings.

A further example of a plant-produced fungicide is tomatin. Irving, Fontaine, and Doolittle (12) isolated from tomato leaves a material which they named lycopersicin, toxic to certain pathogenic fungi. This substance, isolated in crystalline condition from *Lycopersicum pimpinellifolium* and since recovered from other tomato species by Kuhn and his associates (14, 15) and by Fontaine *et al.* (8), was renamed tomatin. It is a glycoside, being a tetrasaccharide of the aglycone tomatidine, for which structure I was proposed by Kuhn, Löw, and Trischmann (16, 17).

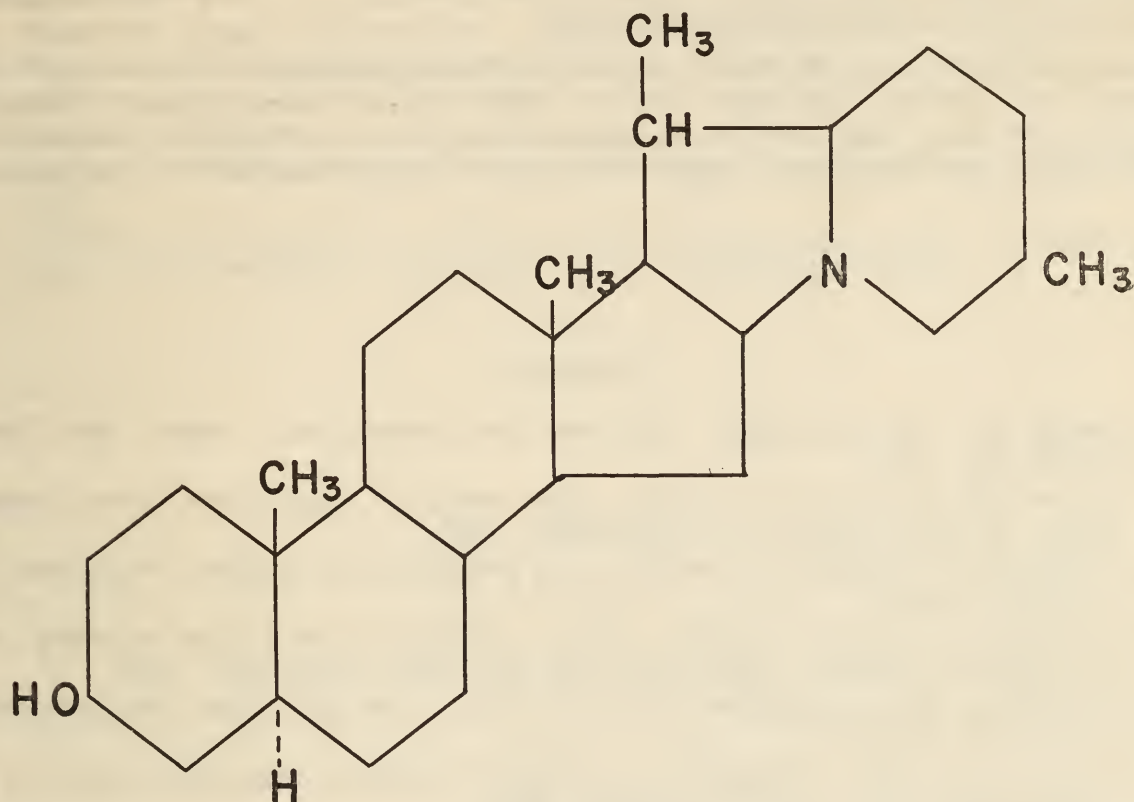
Although tomatin effectively inhibits the growth of *Fusarium lycopersici* Sacc. in pure culture (12) and has been described by Gäumann (10) as a preformed resistance factor, no direct evidence has been found that it is associated with the resistance of certain tomato varieties to *Fusarium* wilt; indeed, Irving, Fontaine, and Doolittle (12) recovered it from susceptible varieties. But it is a compound of interest because of its close relationship to demissidine (II) and solanidine (III), the aglycones of the glycosides with which Kuhn and his colleagues associated the resistance of *Solanum* spp. to the Colorado potato beetle (see p. 26).



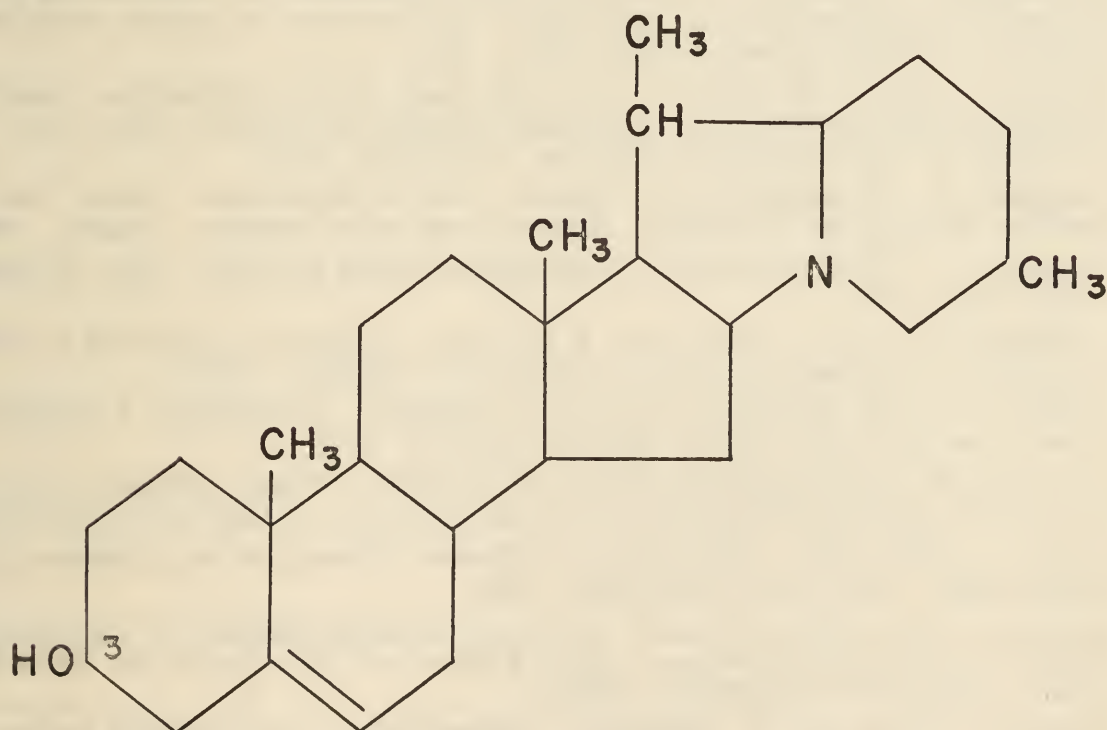
I TOMATIDINE

The heartwood of many timber trees is resistant to fungal decay. In part this greater durability over sapwood is due to the absence of organic materials such as starch and fats readily broken down by microbial enzymes. From the heartwood of many conifers, however, strong antifungal compounds have been isolated. Pinosylvin (3,5-dihydroxy*trans*stilbene), for example, is some 5 to 50 times as toxic as phenol to wood-rotting fungi (20); and the pinosylvin resins, detected by the blood-red coloration produced by bisdiazotized benzi-drine, are present in the heartwood of many pines. Schmitz (quoted by

Sowder, 22) isolated, by acetone and alcohol extraction, a fungicidal resin which he considered a cause of the unusual durability of the timber of the western red cedar (*Thuja plicata* Donn). This tree, chosen by the Pacific Coast



II DEMISSIDINE



III SOLANIDINE

Indians for their totem poles not only because of size but because of durability, contains in its heartwood a remarkable series of antifungal phenols (6). These are the thujaplicins, the α -, β - and γ -isopropyl derivatives of tropolone (2-hydroxycyclohepta-2,4,6-trienone).

The fungicidal action of these three compounds on blueing and decay fungi was shown by Rennerfelt (21) to be about as high as that of sodium pentachlorophenate. The chemistry of these and other constituents of heartwood of conifers have been reviewed by Erdtman (7) who also discusses their physiological and taxonomic significance.

Another stilbene derivative, 2,3',4,5'-tetrahydroxystilbene, was isolated by Barnes and Gerber (2) from the heartwood of the Osage orange, *Toxylon pomiferum* Raf. The toxicity of this compound to a cellulolytic fungus was such that it was considered responsible for the decay resistance of this timber.

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Chapter 5

HIGHER PLANTS *vs.* BACTERIA:

Being Mainly Concerned with the Rhizosphere

The influence of plant roots on soil microorganisms was recognized as long ago as 1904 when Hiltner (2) coined the term "rhizosphere" for the region around the plant roots that supports a microbial population which differs both quantitatively and qualitatively from that of the bulk of the soil. The nature of the flora of the rhizosphere is in part determined by the crop plant, as was shown by Wallace and Lochhead (12), and indicates a preferential stimulation of those bacteria that require amino acids for maximum growth (4).

The source of the nutrient responsible for the greater bacterial numbers of the rhizosphere may range from excretory products of the living roots to the products of autolysis and breakdown of dead roots or of the sloughed-off root fragments. That dead roots are not the responsible source is indicated by the results of Vozniakovskaia, reported by Lochhead (5), who found much larger numbers of microorganisms in the rhizosphere of living roots than in that of dead roots, the latter number being hardly different from that of the control soil. Rovira (10, 11) tried to find out whether the source of this root "exudate" was true excretion or the product of autolysis of sloughed-off cells. He found that although the amount of cell debris from both oats and peas is doubled between 14 and 15 days after germination, the amount of material soluble in acetone (containing 10 per cent of 10 N hydrochloric acid) is only increased by half. He concluded that as the plant ages the true root excretion becomes less important than the cast-off cell material in its influence on the rhizosphere population. No confirmation has been found of the early report of Roberts (9) that root hairs are able to burst, releasing the cell contents, and then resume their normal appearance.

On the other hand there is ample evidence that amino acids and even nucleosides are present in nutrient solution in which plants are growing. Lyon and Wilson (6) recovered a relatively high amount of organic matter. West (13) found thiamin and biotin in sterile nutrient solution in which flax seedlings were growing. These vitamins are essential for the growth of microorganisms of widely separated groups, e.g., *Micrococcus aureus* Zopf., of medical importance, and *Rhizobium trifolii* Dangeard, of agricultural importance. Nutman (7) used the color reactions which bentonite gives with certain amino compounds, with vitamin A and with carotinoids to examine the nature of the root exudate. The roots of clover growing on agar slopes containing bentonite yielded color when supplied with nitrate but no color was produced by oats, rye grass, or radish. Fries and Forsman (1), employing the specific reactions of nutritionally deficient *Ophiostoma* mutants and ultra-violet absorption, made a chromatographic examination of these compounds in the exudate from peas and the roots of pea seedlings, which revealed the presence of amino acid and nucleic acid derivatives. Most of the latter consisted of compounds of high molecular weight, mononucleotides and possibly polynucleotides; free purines and pyrimidonucleosides were present in only

small amounts. For these tests Fries and Forsman generally used excised roots for, in experiments with the whole seedlings, the technique did not permit a distinction between exudate from roots and from cotyledons.

Other early evidence of the release from plant roots of materials nutritive to bacteria is summarized by Katznelson, Rouatt, and Payne (3). These workers showed, by chromatographic methods, that appreciable quantities of amino acid and detectable amounts of reducing compounds such as glucose are liberated from a variety of plants. Larger amounts appeared from plants grown in soil that was allowed to dry until the plants wilted and was then remoistened. Rovira (10) identified no less than 22 amino compounds from pea root after 21 days growth, and 14 from oats, the proportions of the various amino acids differing between peas and oats. Fructose and glucose were excreted only during the first 10 days of growth. A perfusion technique was used by Parkinson (8) to demonstrate the excretion of amino acids from oats growing under sterile conditions.

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Chapter 6

THE REACTION OF HIGHER PLANTS TO INSECT ATTACK

Gall formation and other abnormalities in the growth of higher plants induced by the attack of insects and mites are often so startling and conspicuous that a rich scientific literature might be expected. Yet in fact their study has, with the exception of gall formation, not progressed far beyond descriptive stages. Accordingly this chapter will be of limited scope and of necessity confined mainly to the work of a few investigators on the reactions leading to gall formation.

A first step in this discussion must be the exclusion of the three-component system of insect, host plant, and virus, which is involved in the insect transmission of the virus diseases of plants. Information on the biochemical responses of the plant to virus infection is at present meagre and will perhaps not become substantially greater till the nature of the plant viruses is more clearly established. Although it is now known that the properties of a number of these viruses are those of a nucleoprotein, it would be premature to treat them all as ecological chemicals.

Further, in this discussion of the reactions of the plant to insect attack, it is convenient to assume that the toxins or active chemicals that are involved are derived solely from the insect. This assumption serves as a reason for allocating to Chapter 3 those chemicals of probable plant origin that determine host range or host resistance, separating them from those toxins that seem more directly to be products of insect metabolism and are the probable cause of the reactions of the plant to insect attack. An example or two will suffice to clarify the position, and to illustrate the hazards of the assumption.

A characteristic feature of the apple infested with the rosy aphid *Anuraphis roseus* is the cluster of distorted small fruitlets developing from the infested flower truss. Every flower of the truss produces an apple, most of which are parthenocarpic. It is well established that seedless fruit may be produced on several types of crop plant by the application of synthetic growth substances such as α -naphthylacetic acid, but no one has yet succeeded in producing parthenocarpic apples by this means. The nearest to success was Swarbrick (13, 14), who sprayed a frost-damaged plot of the varieties Miller's Seedlings and Cox's Orange with a mixture of growth substances and obtained, on the Miller's Seedling trees, a crop of small apples even though their cores were destroyed by the frost. But as Luckwill (8) pointed out, this fruit may have been pollinated before it was affected by the frost; and it is therefore doubtful whether it provides a true example of parthenocarpic induction. The rosy apple aphid, however, never seems to fail to produce parthenocarpy in the blossom it attacks, and it seems logical to suppose that the auxin level necessary for fruit development is produced by the host plant as a consequence of a stimulus provided by the insect.

"Big Bud" of black currant is the result of the infestation of the bud by the gall mite *Phytoptus ribis* (Westw.) Nalepa, but the abnormal growth is again presumably the consequence of a disturbance of the plant auxin mechanism. In the red and white currant, however, the attack of the mite

rapidly kills the bud—an indication of toxin production. In both examples, rosy aphid and big bud, the intervention of physiologically active chemicals produced by the host plant is due to an initial stimulus which is provided by the pest.

Turning now to gall formation as a result of insect or mite attack, two types of gall have been differentiated. In some cases, as with the gall midges, a well-defined gall is produced with tissue differentiation as complete as that of the normal plant tissue. Such galls Küster (6) named “prosoplasmas,” distinguishing them from the “kataplasmas,” which are galls less highly differentiated than the plant tissues from which they are derived. Prosoplasmic galls tend to be highly specific, and the insect responsible can be foretold from the nature of the gall. The high degree of growth differentiation indicates a most intimate and ordered sequence of the reactions by both the host plant and the larva sheltering within the gall. Attempts to produce such galls by the injection of preparations of the causative insect or by chemicals have not been successful. Boysen Jensen (3) for example, was not able to produce on beech leaves the highly specialized gall that is the result of the attack of the gall midge *Mikiola fagi* Hart., but he obtained tumorous growth on leaves treated with lanolin on which the larva had been placed. He concluded that gall formation is caused by the ability of the larva to produce, in an orderly fashion and at definite places on leaf or in gall, substances similar to growth substances, which induce gall formation and which control its organization.

The opinion that the less highly differentiated kataplastic galls are produced as a result of chemical stimulus goes back to Malpighi who, according to Schlechtendahl (12), considered that the gall-former injected at oviposition a poison that upset the plant metabolism and organization thereby producing malformation. This toxin hypothesis was accepted by many investigators listed by Beck (2). Among the more critical experimental studies is that by Beck himself on gall formation on goldenrod (*Solidago* spp.) by the larva of the moth *Gnorimoschema gallaesolidaginis* Riley. He found that the development of the gall could be curtailed by limiting the area fed upon by the larva, and that the substances stimulating growth of the plant tissue would diffuse into normal stems grafted to the developing galls. Anatomical changes in normal stem could be induced by a silky substance excreted by the feeding larvae, but continuous deposition of this substance over the surface of the larval chamber was necessary for the formation of typical galls. Martin (9) produced stem galls on sugar cane by inoculation with extracts of the leafhopper *Draeculacephala mollipes* Say., of *Peregrinus maidis* Ashm. or of the mealy bug *Trionymus sacchari* Ckll. Galls developed even when the extracts of the first two insects were autoclaved, showing that the toxins responsible are heat-stable. The symptoms they produced apart from gall formation, namely, strong growth from adventitious buds, resemble those of a disarranged auxin balance in the host plant.

A remarkable account is given by Lewis and Walton (7) of the process by which the cone gall of witch hazel (*Hamamelis virginica* L.) is initiated by the aphid *Hormaphis hamamelidis* Fitch. They observed that minute drops of a substance were secreted from glands opening into the stylar canal, and that crystalloids appear in this substance and pass into the nucleolus of the plant cells there to split up and to pass at mitosis to the daughter nuclei. A continued injection of the substance by the aphid is necessary for gall formation to proceed, but Lewis and Walton considered that the injected substance is responsible for the rapid multiplication and re-differentiation of the natural

cells of the young leaf into cells of the gall tissues. The nature of the substance, apart from its staining reactions, which appear to be those of a nucleoprotein, is not discussed, but Lewis (*in litt.*, Jan. 12, 1956) has not yet excluded the possibility that a virus is involved.

A heat-labile chemical is involved in gall production on chestnut oak by the pit-making oak scale *Asterolecanium variolosum* Ratzeburg for although Parr (11) obtained 90 per cent success in inducing gall formation by the injection of extracts of the salivary glands of the insect, none were produced by the extract heated to 60°C., nor were galls produced by the injection of indoleacetic acid. For these reasons Parr concluded that the toxins are enzyme-like.

Several investigators have concluded that the enzymes of gall larva are factors in gall formation. An example is due to Beck (1), who identified amylase, invertase, and a protease, in the excreta of maggots of the stem borer *Eurosta solidaginis* on goldenrod; and he considered that the proteolytic enzymes and an elevated pH are of importance for gall formation by this insect. Other examples are cited by Newcomb (10), who approached the problem of gall formation by a study of the metabolism of gall and normal tissue, for a common feature of many insect galls is the high content of polyhydroxyphenols or their condensation products such as tannins.

The reactions of the host plant to insect attack, even in cases where gall formation is not involved, are often so startling, as in the wilts produced by certain mealy bugs and froghoppers or the catfacing of fruit by some capsids and pentatomids, that their study would provide interesting problems for the biochemist. As an extensive review of the subject matter by Carter (4, 5) is available, there is no need to discuss here further examples of the observational work widely reported in the entomological literature.

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Chapter 7

FUNGI vs. HIGHER PLANTS:

The Role of Phytotoxins in Plant Pathology

"Microorganisms responsible for disease act by virtue of the toxins they produce." This sweeping generalization of Gäumann (21) rests, however, mainly on the evidence of human and animal pathology. Its extension to phytopathology indicates a vast territory for exploration in ecological chemistry, for the fungi predominate among the phytopathogens and as yet little is known of the chemical nature of the toxins involved in the interactions of host plant and pathogenic fungus. And it is doubtful whether there will yet be general agreement with Gäumann's statement that the phytotoxin is produced by the pathogen that causes the host to become diseased. It is certainly safer at this stage to regard the phytotoxin as the product of interaction of host and pathogen, though it is already known that the latter may be capable of excreting toxins into the culture medium in which it is grown.

The search for antibiotics among the metabolic products of fungi growing in culture media has led to the isolation of many active compounds and, even in 1951, Brian (2) was able to list about one hundred. There is, however, no need here to catalogue all the biologically active products of fungal metabolism; it will suffice to select those known to be produced by phytopathogenic fungi and which, when injected into or applied to the host plant, produce symptoms similar to those produced in the diseased plant by the pathogen. To certain of these compounds, Dimond and Waggoner (18) applied the term "vivotoxin," defined "as a substance produced in the infected host by the pathogen and/or its host, which functions in the production of disease but is not itself the initial inciting agent of the disease." The final phrase of this definition is required to differentiate a virus and a vivotoxin, for the former can be regarded as the initial inciting agent, even though a vivotoxin may be involved in its interaction with its host.

The subject of the fungus-produced phytotoxins has a brief history. Whereas the significance of bacterial toxins in animal pathology was promptly established mainly because the toxin is an antigen and produces demonstrable antibodies in the animal, this criterion was not available in the case of the fungal diseases of plants. For the fungal toxins are not antigenic and as late as 1936 many authorities, including Brown (8), doubted whether toxin production *in vivo* had any significance in phytopathology.

The development of the idea that toxins are involved in the etiology of the plant diseases caused by fungi has been well discussed by Brian (3). To his review may be added reference to the more recent work of Forsyth (19a) and of Ludwig and his students (30). Forsyth observed that viable urediospores of the rust fungus *Puccinia graminis* Pers. var. *tritici* Erikss. & Henn held in a closed container germinated and grew poorly in comparison with similar spores in an open container. On the basis of ultraviolet absorption data, he deduced that the self-inhibitor was trimethylethylene, which he found to inhibit germination at vapor concentration of 200 p.p.m. Forsyth suggested

that the trimethylethylene produced within the host plant tissue by the metabolism of the fungus was a phytotoxic factor associated with host resistance.

Ludwig *et al.* (30) demonstrated that the induction of the seedling blight phase of barley inoculated with *Helminthosporium sativum* is associated with the production by the fungus of toxic substances that are not only prerequisite for the infection of the host tissue by the pathogen, but that may predispose the roots and basal parts of the plants to attack by other microorganisms not normally regarded as pathogens.

Turning now to the consideration of those phytotoxins which have received detailed study, the first, alphabetically, is alternaric acid.

Alternaric Acid:

Alternaria solani (Ell. & Mart.) Jones & Grout causes early blight of solanaceous plants, a disease in which a collapse of the plant tissue precedes the actual infection of that tissue by the fungus. For this reason a diffusible toxin was thought to be involved and, in 1949, Brian and his colleagues (4) reported the isolation, from liquid media cultures of the fungus, of a substance, alternaric acid, which, when added to nutrient solution in which tomato, pea, cabbage, and other plants were growing, produced a severe wilt of the plants. Darpoux, Faivre-Amiot, and Roux (14) also obtained a biologically active material, which they named alternarin, from cultures of a strain of *A. solani*. The same authors, with Ridé (15), described alternarin as a hard colorless gum but slightly soluble in water and retaining some activity even after autoclaving at 120°C. They obtained no damage to tomato plants sprayed with a solution containing 100 mg. alternarin per liter, but they recorded the inhibiting effect of alternarin on the growth of a number of pathogenic fungi.

Alternaric acid was also isolated by Pound (35). The chemical study of Grove (25) showed it to be a dibasic acid of formula $C_{21}H_{30}O_8$, and of m.p. 138°C. forming a monohydrate of m.p. 135°C. It contained one carboxyl group whereas the second acid group appeared to be a β -dicarbonyl grouping, for a chloroform-soluble copper complex is formed. Little further on its molecular structure has yet been published.

Although alternaric acid is able, at extreme dilutions, to produce in tomato many of the symptoms of early blight, there is doubt whether it can be held responsible for the whole symptom syndrome. Other toxins are almost certainly involved and were isolated by the French workers. Moreover Brian and co-workers (5) found that only two of twelve strains of *A. solani* produced alternaric acid, and that these two strains were the least pathogenic. It would, therefore, be expected that the etiology of the disease is complicated, sufficiently to mask a direct correlation between severity of symptoms and the ability of the pathogen to produce toxin in nutrient culture.

Diaporthin:

The sweet chestnut *Castanea dentata* (Marsh.) Borkh. was virtually eliminated from North America by the blight caused by *Endothia parasitica* (Murr.) And. This fungus was shown by Bazzigher (1) to produce in culture a substance, named diaporthin, which was toxic not only to the European species of chestnut *C. sativa* Miller but to several other species of higher plants and which induced a wilt of tomato. The chemistry of diaporthin is being studied by A. Boller, but no details of this work have yet been published.

Fusaric Acid:

In early work on the biochemistry of the "Bakanae" disease of rice (see below), Yabuta, Kambe, and Hayashi (55) isolated from cultures of the fungus a growth-restraining substance, which they named fusarinic acid, and which they showed was probably a butylpicolinic acid. Unlike gibberellic acid (see below), which produces elongated growth, fusaric acid (as it is now called) was found by Yabuta and Hayashi (54) to be extremely phytotoxic. Confirmation of its structure as 3-*n*-butylpyridine-6-carboxylic acid was obtained by Tamari (42), who synthesized 3-butylpyridine, and showed that it was identical to fusarinin obtained by the decarboxylation of fusaric acid.

Fusaric acid was also identified by Gäumann, Naef-Roth, and Kobel (23, 24) among the several toxins produced by *Fusarium lycopersici*. A characteristic of its phytotoxic effect on the tomato, cotton, and other plants was an intercostal necrosis of the foliage. The inhibition of growth of rice seedlings by fusaric acid was attributed by Tamari and Kaji (43) to an inadequate synthesis of iron- and copper-containing enzymes caused by the immobilization of these metals as chelated compounds.

Gibberellins:

A symptom, unusual in the physiological consequences of fungal attack on plants, is the stimulation of growth induced in vigorously growing plants by certain spp. of *Fusarium*. Seedling rice plants, if not too severely infected by *Gibberella fujikuroi* (Saw.) Wollenw., the conidial stage of which is known as *Fusarium moniliforme* Sheld., become etiolated and grow taller than those not infected. An infected crop therefore presents the appearance of uneven germination and growth, a characteristic of the "bakanae" (elongation) disease of rice. The increased height is due to a greater length of stem internode; but in maize and other cereals, as well as rice, De Haan (16) found that there is also an increase in leaf growth and in the production of dry matter.

The observation of Kurosawa (27) that the bakanae effect can be produced by watering rice seedlings with the cell-free filtrate from liquid cultures of *G. fujikuroi* was quickly confirmed by other Japanese workers, and two active principles, gibberellins A and B, were isolated by Yabuta and Hayashi (52) from the crude amorphous powder that they prepared from the culture media. Gibberellin A crystallized from ligroin ethyl acetate in long prisms of m.p. 194-196°; gibberellin B in short prisms melting with decomposition at 245-246°C. In later work Yabuta and his colleagues (56) apparently reversed the lettering; gibberellin A being assigned to a substance crystallizing in short colorless columns insoluble in benzene and decomposing at 242-244°, $[\alpha]_D +36.1$ and of molecular weight corresponding to $C_{22}H_{26}O_7$; gibberellin B to the compound soluble in benzene and giving long columns melting at 197-199°, $[\alpha]_D -82.3$ and of molecular weight corresponding to $C_{19}H_{22}O_3$. Gibberellin A was more potent than gibberellin B in inducing the elongation of rice seedlings and was readily converted to gibberellin B by the action of dilute acids.

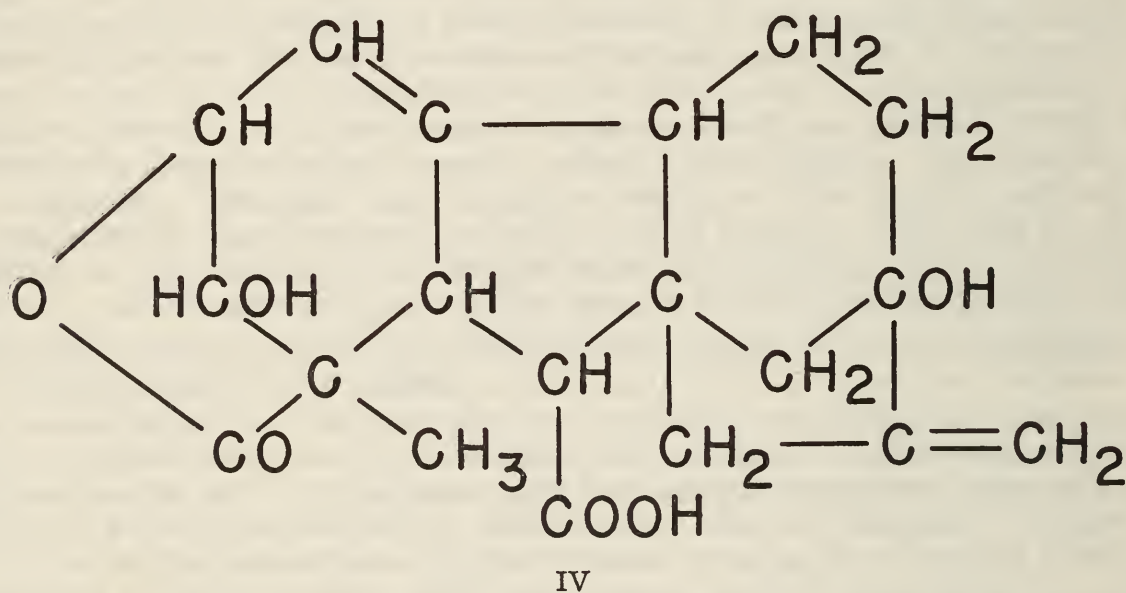
The interesting effect of gibberellin on plant growth prompted a repetition of the Japanese work both in Great Britain and in the United States. In the former country, Curtis and Cross (13) reported yields, up to 180 mg./l. from Raulin-Thom medium containing sucrose as carbon source, of an active principle which they purified by adding an equal volume of light petroleum to a solution in boiling ethyl acetate, obtaining crystals decomposing at

233-235° and of rotation $[\alpha]_D^{44} + 82^\circ$. As the crystals were of a monobasic acid of molecular weight $C_{19}H_{22}O_6$ the compound was named gibberellic acid, which was shown to be not identical to gibberellin A.

In the United States, Stodola and his colleagues (41) recovered from 160 U.S. gallons of a medium containing mineral salts and $1\frac{1}{4}$ — $1\frac{1}{2}$ per cent glucose, up to 12 grams of crude crystalline gibberellin which was a mixture of gibberellin A ($[\alpha]_D + 36^\circ$) and a compound ($C_{19}H_{22}O_6$, $[\alpha]_D + 92^\circ$) which they named gibberellin X. The identity of the latter product with gibberellic acid was established by Cross (12).

Under mild conditions of acid hydrolysis gibberellic acid was found by Cross to yield *allogibberic* acid ($C_{18}H_{20}O_3$, m.p. 200.5-203°, $[\alpha]_D - 80^\circ$). Under these conditions gibberellin A forms gibberellin B, which Cross found to have an infrared spectrum identical to that of *allogibberic* acid. This acid is isomeric and closely related to gibberic acid, which is a main product of the hydrolysis of gibberellic acid by boiling mineral acid. Cross showed that gibberic acid is a tetracyclic keto-acid containing an aromatic ring. The hydrogenation of gibberellic acid and of the two gibberic acids yields a hydrocarbon, gibberene and a ketone, gibberone, as shown by Yabuta and his co-workers (57). These compounds were shown by Mulholland and Ward (32) to be 1,7-dimethylfluorene and 1,7-dimethylfluorenone respectively.

From these reactions Cross and his colleagues (12a) deduced that gibberellic acid is of structure IV



though the points of attachment of the lactone group are in doubt.

The physiological effects of gibberellin treatment on seedling plants are similar to the effects of the pathogen on rice seedlings. Brian *et al.* (6) showed that, in treated wheat plants, stem internodes are longer and the leaves longer, narrower, and paler than those of untreated plants. Pea seedlings developed longer internodes, but the leaves were of a size similar to those of control plants. In contradistinction to the Japanese workers (58), who found little difference in the total weight of treated and untreated rice seedlings, Brian and his colleagues (6) found treated plants had a greater dry weight, particularly of shoot, than the control plants. They attributed this increase to a greater carbon assimilation, for it was in part reflected in a greater content of soluble carbohydrates, particularly of glucose. The longer internodes of the treated plants is in part explained by a lengthening of the cell of the stem

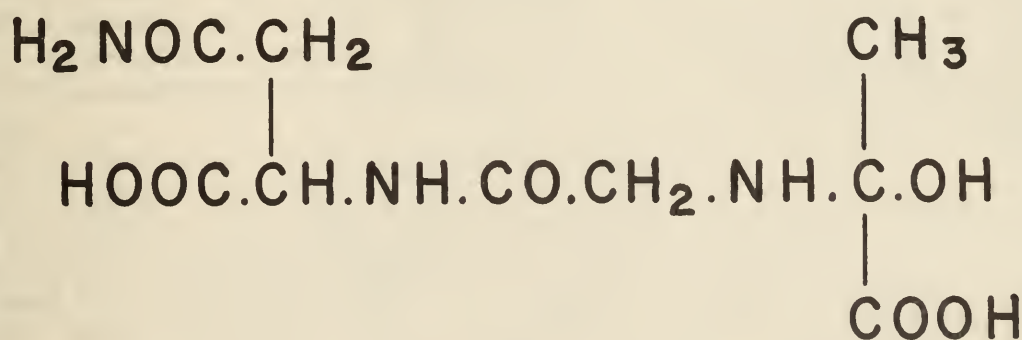
internodes; and though Yabuta and Hayashi (53) considered that cell multiplication increased following treatment, neither they nor Brian and his colleagues provided evidence on this matter. In peas, however, the latter investigators found the increase in cell length sufficient to account for the greater internode length. In this respect, therefore, gibberellic acid resembles indoleacetic acid. Yet neither the Japanese nor the British workers were able to obtain the typical auxin reaction of epinasty with gibberellic acid, which was also inactive in the standard *Avena* and Went pea tests. Brian and Hemming (7) do, however, record a curious effect in dwarf forms of peas, of broad beans (*Vicia*), and of French beans (*Phaseolus*). Suitable doses of gibberellic acid virtually eliminate the difference in growth rate between tall and dwarf varieties.

Clearly *Gibberella fujikuroi* produces metabolic products of extreme importance in the study of plant growth, a study which is all the more interesting because of the contrast between promotion of internodal growth by gibberellic acid and its suppression by maleic hydrazide.

Lycomarasmin:

One of the first chemical compounds to be isolated from the culture media of a fungal pathogen, and which is capable of producing disease symptoms in plants treated with it, was lycomarasmin. This substance was recovered from cultures of *Fusarium lycopersici* Sacc. by Clauson-Kaas and Gäumann (10), and was named by them. Excised tomato leaves, when placed in a 0.02 per cent solution of lycomarasmin, acquired symptoms of the wilt characteristic of infection by *F. lycopersici*. Wilt developed more rapidly if a trace of ferric iron was added with the lycomarasmin.

On hydrolysis lycomarasmin, a white powder melting with decomposition at 227-229°C., yields glycine, ammonia, aspartic and probably pyruvic acid. It is therefore a peptide, and Woolley (51) deduced that its structure is *N*-(α -(α -hydroxypropionic acid))-glycylasparagine (V). It readily chelates



V

with ferric iron and with copper. Gäumann (20) considered that the iron-complex is the effective toxin in tomato, for it is about ten times as toxic as lycomarasmin itself.

There are, however, a number of reasons for a search beyond lycomarasmin for the explanation of the syndrome of *Fusarium* wilt. The iron-lycomarasmin complex fails to produce with sufficient rapidity and completeness the full symptoms of the disease. Lycomarasmin has not been recovered from the diseased plant, and it appears in the culture medium only after a time interval which, Dimond and Waggoner (17), suggested, indicates that it is a product of lysis rather than of active growing mycelium. Both wilt-susceptible and

wilt-resistant varieties of tomato are equally sensitive to lycomarasmin, as shown by Gäumann, Naef-Roth and Miescher (22). Scheffer and Walker (36) could find no differences in disease intensity between normal and iron-deficient tomato, though such differences would be expected if the iron-lycomarasmin complex were an important symptom producer. Moreover, the copper lycomarasmin complex is more stable than the iron complex and is much less phytotoxic, whereas 8-quinolinol will decompose the iron-lycomarasmin complex; yet neither copper nor 8-quinolinol will alleviate the disease (Waggoner and Dimond, 44). For these reasons the role of lycomarasmin as the primary factor in the pathology of tomato wilt is suspect.

Certainly *F. lycopersici* is able to produce, in artificial culture, biologically active compounds other than lycomarasmin and the previously mentioned fusaric acid. Some of these biologically active compounds were isolated by Plattner and Nager (34) and by Cook and his colleagues (11) from cultures of several species of *Fusarium*, but no evidence is given that these compounds are phytotoxic.

Pectolytic Enzymes:

The immediate cause of wilt in the diseased tomato was shown by Ludwig (29) to be a plugging of the vascular system. Scheffer and Walker (36) found that a similar plugging and a vascular browning typical of the diseased plants could be produced by placing tomato cuttings in dilute filtrates from young cultures of the pathogen. Winstead and Walker (48) continued this study and found that filtrates from cultures of various wilt-producing species of *Fusarium* had relatively high pectin methylesterase and low polygalacturonase activities, and all were capable of producing vascular browning and plugging in cotton, tomato, cabbage, and pea plants. The role of the extracellular pectic enzymes of *F. lycopersici* was studied by Waggoner and Dimond (45), who were non-committal on their phytotoxic activity because of the difficulty of ensuring freedom of the enzymic preparation from contamination.

Evidence supporting the idea that pectic enzymes are involved in pathogenicity was obtained by Scheffer, Gothoskar, Pierson, and Collins (37), who showed that the culture broth of *Verticillium albo-atrum* Reinke & Berth. contained much polygalacturonase but little pectin methylesterase. This pathogen likewise produces a wilt in the infected host plant which provides an interesting contrast to the *Fusarium* wilts.

There is, however, little doubt that these enzymes are involved in the physiology of fungal parasitism, a subject of many years' study by Brown and his students, and reviewed by Brown (8, 9). Brian (3) accepts tentatively that the vascular plugging is due directly or indirectly to the reactions of the pectin methylesterase and glucosidases of the infecting fungus, and that wilt is the consequence of this vascular plugging. This hypothesis readily explains the marked similarity in symptoms of the various wilt diseases.

It is a common fate of those who are unhappy until they have erected a tidy pigeonhole-like classification to become entwined in the lines of distinction they seek to establish. Dimond and Waggoner (18) found no difficulty in classifying the extracellular enzymes as vivotoxins. The general acceptance of enzymes such as the lecithinases of snake venom as toxins is perhaps associated with the drastic haemolysis produced by extremely low doses. But when the enzyme is one whose function it is to assist in the breaking down of large organic molecules to fragments that are more easily moved through membranes, a toxin classification seems inappropriate. It would, for example, embrace the enzymes of the insectivorous plant, whereby it digests the entrapped insect, and the enzymes secreted into the alimentary canal, for the

contents of the gut are in a strict sense exterior to the animal. Such phenomena belong to the chemistry of digestion rather than to toxicology. Yet the significance of the hydrolytic exoenzymes in the etiology of plant disease caused by fungi and bacteria is clear from the work, among others, of Brown and his students (8, 9).

The hazards of treating parasite and host as if the results of their interaction were separate and unrelated phenomena have already been stressed. These risks have been taken in this consideration of the relation of fungal pathogen and host plant because of the evidence that the toxin is a metabolic product of the pathogen.

The close resemblance, in so many cases, of disease symptoms and of effects produced by plant growth substances is a certain sign that one result of the pathogenic attack is a disturbance of the growth mechanism of the host. In the case of tomato wilt, for example, a characteristic symptom first observed by Wellman (46) is an epinasty, a typical plant response to ethylene and an early result of the application of synthetic growth substances such as 2,4-D. Dimond and Waggoner (19) showed that ethylene is produced both by *F. lycopersici* in culture and by the infected tomato plant, in amounts sufficient to account for the observed epinasty. But ethylene is normally produced at least in small amounts, particularly by ripening fruit (see page 11), and may be liberated in much larger amounts by mechanically injured plants as shown by Williamson (47).

Evidence is forthcoming also that the natural auxin β -indoleacetic acid is involved. This acid was isolated by Wolf (49) from cultures from *Ustilago zaeae* Unger, the cause of smut of maize, a prominent symptom of which is a gross malformation of the ear resembling that produced by the direct application of indoleacetic acid. Other experimental demonstrations of the production of indoleacetic acid by fungi in artificial culture, particularly when tryptophan is added, are summarized by Pilet (33), who showed that the leaves of *Euphorbia cyparissias* L. parasitized by *Uromyces pisi* Wint. contained about a hundred times more indoleacetic acid than healthy leaves. Wolf (50) quoted the review of Hirata, who showed that the auxin contents of leaves attacked by three different rust fungi were 1.6, 5.0 and 13.4 times that of comparable healthy tissues. Wolf further showed that the cedar apple rust fungus *Gymnosporangium juniperi-virginianae* Schw. produced, in culture, concentrations of up to $1.1 \times 10^{-4}M$ of indoleacetic acid, and he suggested that the high level of auxin found in rust-infected plant tissue may be attributed entirely to indoleacetic acid synthesis by the pathogen.

Yet another factor responsible for the close resemblance between symptoms produced by fungal pathogens and those produced by auxin accumulation may be a disturbance of the normal auxin metabolism of the host plant. The coffee leaf disease, caused by *Omphalia flavida* Maubl. & Rang., results in a severe defoliation which Sequeira and Steeves (38) ascribed to the ability of the fungus to produce in the plant, as in culture media, a heat-labile, non-dialysable, pH-sensitive auxin-inactivating substance. As this substance is inactivated by ascorbic acid and by cysteine, and is sensitive to cyanide and to hydroxylamine, it is considered to be an oxidative enzyme. The authors suggest that it is responsible for the main pathogenic effects of the fungus.

Mycorrhizal Relations:

Although many higher plants enter into mycorrhizal association with fungi, the phenomenon is still little understood. The profound modification of the plant roots by a profuse dichotomous branching of the rootlets is strongly suggestive of the intervention of plant growth substances. MacDougall

and Dufrénoy (31) showed that auxin occurs abundantly in the fungal hyphae of pine mycorrhizae. Slankis (39, 40) demonstrated that the mycorrhizal fungi, *Boletus variegatus* and *B. luteus*, exuded a substance causing a dichotomous branching of isolated pine roots, and that the auxins β -indoleacetic acid and α -naphthylacetic acid exerted a similar effect on both isolated and intact pine roots. Hacskeylo and Palmer (26) and Hacskeylo (*in litt.* Mar. 20, 1956) also found that indoleacetic acid causes proliferation of the roots of pine and other trees on which the mycorrhizal fungus is of the ectotrophic type, i.e., on which the fungus mycelia form a mantle over the rootlets and rarely penetrate into the root cells. On the other hand, the roots of trees and shrubs with which the mycorrhizal association is of the endotrophic type, i.e., on which the fungal hyphae penetrate into the plant cells, do not proliferate on treatment with indoleacetic acid. Levisohn (28), however, obtained a forking of the roots of plants with intracellular (haustorial) mycorrhizal infection by watering the seedlings with leachings from pots in which the seedlings had established mycorrhiza.

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*Title translated from Japanese.

Chapter 8

FUNGI vs. FUNGI:

The Chemical Basis of Biological Control

A first explanation of the antagonistic effects of one fungus species upon a second is competition for essential nutrients. That this explanation may not be adequate became apparent when it was found that the application of nutrient, in the form of green manuring, was a method of controlling potato scab. Sanford (66), in 1926, thought that the reduction in potato scab achieved by plowing in green rye was due to the inhibitory influence of certain predominant microorganisms on the development of *Actinomyces scabies* (Thaxt.) Skinner, the pathogen responsible for potato scab. In 1927 Millard and Taylor (53) confirmed, experimentally, that the enrichment of the organic matter of the soil encouraged the development of fast-growing saprophytic Actinomycetes and that the addition of the latter to soil infested with *A. scabies* suppressed the development of potato scab.

An example from the fungi arose the following year, 1928, when Machacek (49) reported on his studies of the association of phytopathogens. He found, in the storage rots of fruits and vegetables, that, though saprophytic fungi may invade host tissue already attacked by a parasite fungus, it was rarely that two species of pathogenic fungi were found at the same time on the same host. By transferring the fungi to culture media, Machacek was able to distinguish between free association of two organisms and complete inhibition of the growth of one by the other. Thus *Penicillium expansum* Link permitted the growth of *Sclerotinia fructicola* (Wint.) Rehm; *Cladosporium fulvum* Cooke stopped the growth of a species of *Botrytis*. Because this inhibition was not affected by the addition of fresh nutrient or a vegetable extract nor by neutralization of the acidity of the medium, Machacek concluded that the surviving fungus produced some toxic substance other than acid.

Vasudeva (71) employed apple tissue in place of nutrient media and found that the ability of *Sclerotinia fructigena* Aderh. & Ruhl. to rot apples is markedly reduced when *Botrytis allii* Munn was also present in the inoculum. A like reduction appeared when the living fungus was replaced by stale media from *B. allii* cultures. She considered that this reduction in growth was akin to "staling," a term frequently applied at that time to the weak growth of an old fungal or bacterial culture.

Turning now to the inter-relationships of soil fungi, the idea, that factors other than competition for nutrient and the "staling" effect of their metabolic products are involved, was first emphasized by Sanford and Broadfoot (67). They found that the pathogenicity of *Ophiobolus graminis* Sacc., a fungus responsible for a footrot of wheat, may be strikingly suppressed by certain soil fungi and bacteria. As the reduction effected by living cultures was more marked than that by the filtrates of old cultures, they concluded that it was due to toxins produced by the living cultures.

Phytopathologists were by 1931 universally interested in the pathogenicity of mixtures of pathogens, and the plea by Fawcett (28) for more attention to this subject was quickly reinforced by the work of Weindling (75). He

examined the methods by which the soil fungus *Trichoderma lignorum* (Tode) Harz suppressed the activities of several soil fungi pathogenic to citrus seedlings.

For historical completeness it may be mentioned that the greater dry rot resistance of timber that had become waterlogged during transfer to the mill was attributed by Falck (27) to its infection by *T. lignorum* and *T. viride*. He suggested that the enzymes of *Trichoderma* are poisonous to wood-destroying fungi. Weindling followed up his conjecture that the yellow crystals he observed in his cultures were active toxins and, with Emerson (79), was able to announce the isolation of a potent fungicide, subsequently named gliotoxin, the first of a large number of biologically active products of microorganisms now generally known as antibiotics.

The impetus given to the study of antibiotics by the successful development of penicillin and streptomycin for medicinal use has resulted in the discovery of innumerable biologically active metabolic products of bacteria, actinomycetes and fungi. Rarely has a field of science shown such rapid expansion as this branch of ecological chemistry and, indeed, the very term "antibiotic" which was defined as recently as 1947 by Waksman (73) as "the metabolic product of a microorganism toxic in low concentrations to other microorganisms," has expanded almost to the deletion of both of the "micro" qualifications. The subject has been amply reviewed, e.g., by Brian (8), and attention need be directed here only to those compounds produced by pathogenic fungi that may be involved in biological control or may prove of practical value for purposes of crop protection.

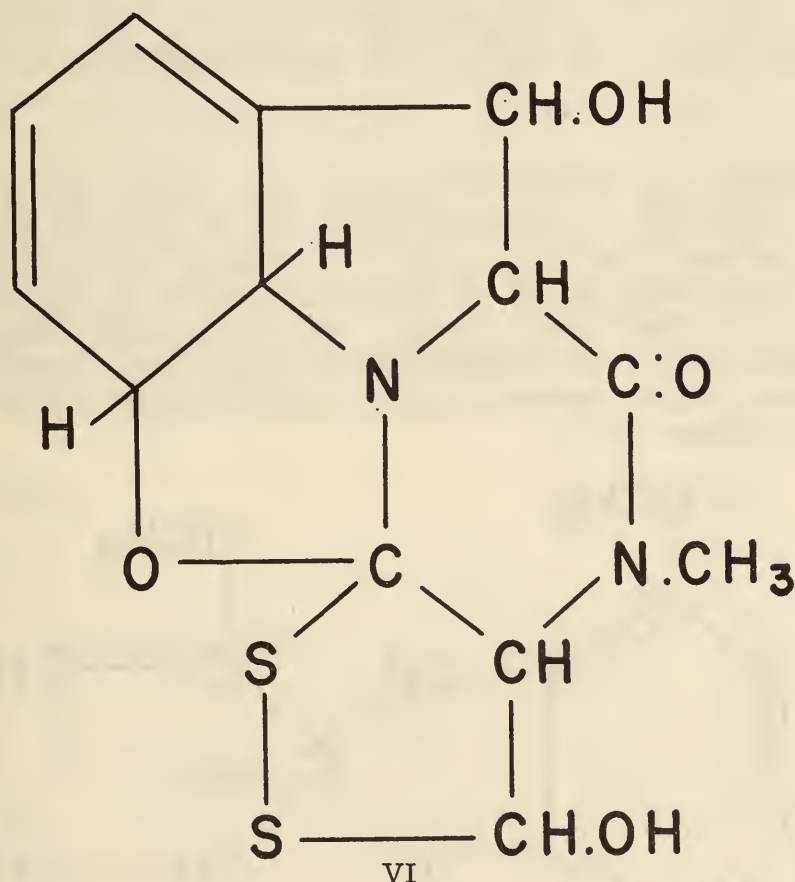
Gliotoxin:

The report of Weindling (75) that the soil fungus *Trichoderma lignorum* parasitized several fungal pathogens responsible for the damping-off of citrus seedlings has been mentioned above. At the surface of the culture medium, the *Trichoderma* hyphae coiled around the hyphae of a strain of *Rhizoctonia solani* Kühn, isolated from diseased citrus seedlings, causing a rapid cessation of growth in *Rhizoctonia* with the release of numerous sheaves of yellow needle-like crystals. Below the surface of the medium the effect of the parasite was less localized and, as the *Trichoderma* colony extended, the host hyphae were killed in advance of the invading parasite and again masses of dark yellow star-like crystals were produced. He (76) quickly established that parasitism was associated with the production by *Trichoderma* of a "lethal principle" which is very unstable except under acid conditions. With Emerson (79) he isolated the crystalline compound by extraction of the culture medium with chloroform, the best yields being up to 70 mg. per liter of filtrate. From analysis and molecular weight determinations the formula $C_{14}H_{16}N_2O_4S_2$ was assigned to the compound. Treatment with aqueous potassium hydroxide rapidly splits out sulphur in a form giving a black precipitate of lead sulphide on the addition of a few drops of a lead acetate solution. The extreme instability of the compound under alkaline conditions was emphasized, but it was noted that it was recovered unchanged from mild acid treatment.

Weindling (77) considered that the fungus forming this yellow crystalline toxin was a species of *Gliocladium*—possibly *G. fimbriatum* G. & A., and not, as he had previously reported (79), a *Trichoderma*. Consequently, at the suggestion of J. R. Johnson, Weindling (78) christened the toxin "gliotoxin." He also found that this same toxin was produced by certain, but not all, isolates of *Trichoderma*. Weindling's identification was subsequently questioned by Brian (5), who also found that gliotoxin was produced by *Trichoderma*

viride (Pers.) Fr. which by then had been accepted as a name of *T. lignorum*. Gliotoxin has since been found to be a metabolic product of *Aspergillus fumigatus* Fres. (52) and its mutant, *A. helvola* Yuill (33). Johnson, its godparent, with McCrone and Bruce (45), identified as gliotoxin a product from cultures of an unspecified *Penicillium*, and in the following year it was found to be produced by *P. obscurum* Biourge (54) and by *P. jensenii* Zal. (16), later corrected to *P. terlikewskii* Zal. (6).

The chemistry of gliotoxin is the subject of a series of papers by Johnson, Dutcher, and their co-workers, in the first of which (42) the molecular weight was revised to $C_{13}H_{14}N_2O_4S_2$. Treatment with phosphorus and hydriodic acid in acetic acid removed the sulphur and two of the oxygen atoms as hydrogen sulphide and water. The determination (24) of the structure of the resultant product as 2,3-dimethyl-1,4-diketotetrahydropyrazino-[1,2a]-indole gave the complete carbon and nitrogen skeleton of gliotoxin. Treatment of gliotoxin with metabolic potassium hydroxide gave a yellow crystalline product retaining one sulphur atom but with the loss of two carbon atoms. The structure of this degradation production was confirmed by synthesis (43) to be a thiohydantoin related to 2-indolecarboxylic acid, namely, 2-thio-3-methylindolo-1',2'-1,5-hydantoin—a conclusion previously reached by Elvidge and Spring (26).



The disposition of the sulphur atoms was revealed by the observation of Dutcher, Johnson, and Bruce (25) that both are removed from gliotoxin in methyl alcoholic solution by aluminium amalgam to give a desthiogliotoxin that showed, by the Kühn-Roth method, a terminal hydroxymethyl group not present in gliotoxin (44). For these reasons, the pentacyclic structure VI was assigned to gliotoxin.

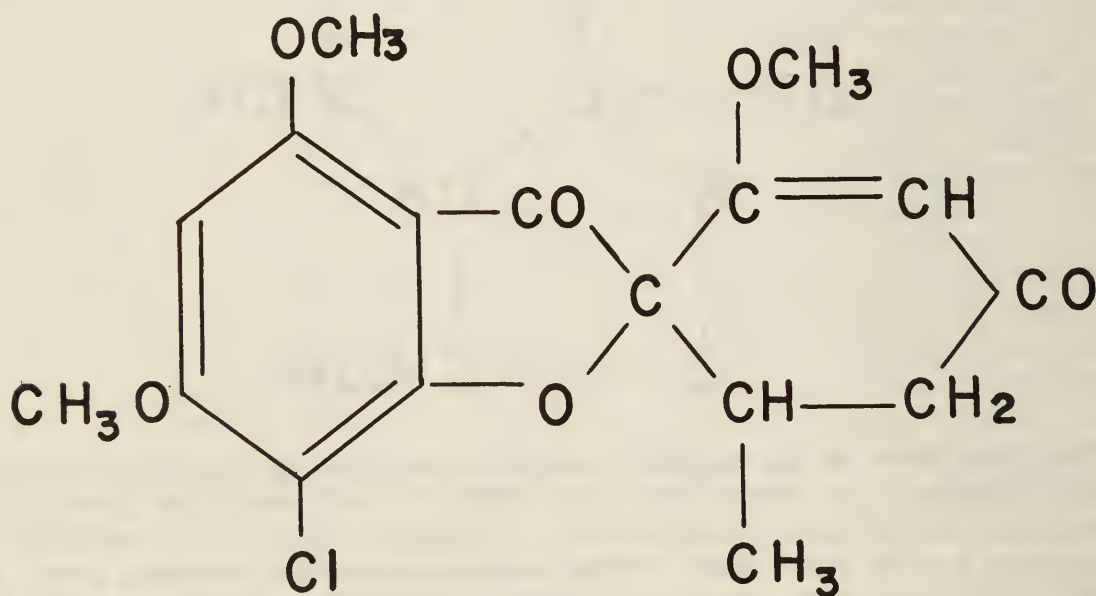
The disulphide bridge of gliotoxin is responsible for the extreme instability of the molecule, especially under mildly alkaline conditions. It is, moreover, associated with the high toxicity of the compound to bacteria and fungi, for

its destruction leads to compounds almost devoid of bacteriostatic and fungicidal activity. The biological properties of gliotoxin are reviewed by Johnson *et al.* (42) and by Brian and Hemming (15); as a fungicide it is about two-thirds as active as mercuric chloride but is selective in its action; at 10 mg./l. it inhibits the growth in broth of the pathogenic bacteria examined; it has an appreciable contact aphicidal action, and intravenous or intraperitoneal injections of about 50 mg./kg. are lethal to rabbit, rat, and mouse. In Brian and Hemming's trials no evidence of phytotoxicity was seen, and the authors considered the possible use of acid formulations of gliotoxin for the control of plant diseases.

In 1945 Brian and McGowan (18) reported the isolation, from some pigment-forming strains of *Trichoderma viride*, of another higher fungistatic substance which they termed viridin. Yields of the order of 45 mg./l. were obtained of colorless rod-like prisms which inhibited the growth of spores of *Botrytis allii* at 0.006 $\mu\text{g./ml.}$ (cf. gliotoxin 3.0 $\mu\text{g./ml.}$) but had little bacteriostatic activity (10). Viridin is extremely unstable except in solutions of pH 3.5 or less. Vischer, Howland, and Raudnitz (72) concluded that the molecule of viridin corresponded to $\text{C}_{19}\text{H}_{16}\text{O}_6$. By chromatography they separated two forms, α and β , differing in optical rotation and in melting point; α -viridin decomposes at 208—217°, β -viridin melts at 140° and is present in small amounts. The latter form is the less potent fungicide, requiring 0.16 $\mu\text{g./ml.}$ to inhibit the germination of spores of *B. allii*, compared with 0.019 $\mu\text{g./ml.}$ for α -viridin.

Griseofulvin:

When studying the failure of conifers on a sandy heath soil, Brian, Hemming, and McGowan (16) found that the soil molds were almost entirely species of *Penicillium*, one of which *P. janczewskii* produced, in artificial culture, a substance that caused an abnormal curling of the germ tubes of *Botrytis allii* (11). This "curling factor" was subsequently found by Brian, Curtis, and Hemming (14) to be identical to griseofulvin, a metabolic product of *P. griseofulvin* Dierckx, isolated and described ten years earlier by Oxford, Raistrick, and Simonart (57).



VII

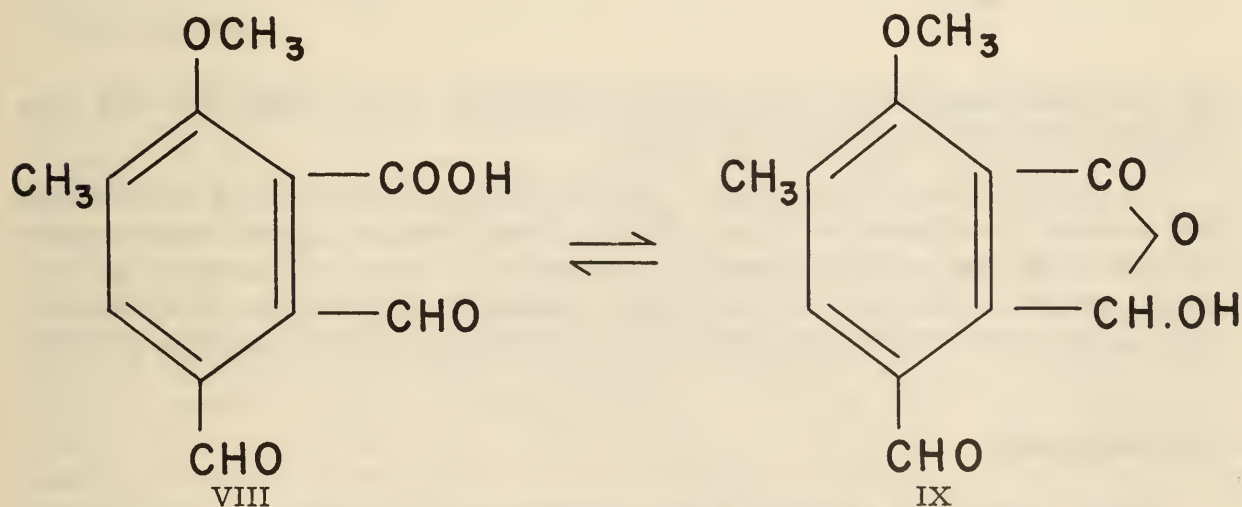
The molecular structure of griseofulvin was deduced by Grove and his associates (38) to be 7-chloro-4,6-dimethoxycoumaran-3-one-2-spiro-1'-(2'-methoxy-6'-methylcyclohex-2'-en-4'-one) (VII).

Two features of this structure are rather uncommon in a natural product, namely, the chlorine substitution in the aromatic ring, and the spiran structure. Obtained in colorless crystals of m.p. 220—221° from ethanol or benzene, it is but slightly soluble in water, giving an optically active solution neutral in reaction and stable in the range pH 3 to 8.8. These data have been subsequently revised in the "Report on Griseofulvin" published in 1955 by the Glaxo Laboratories, Ltd., to m.p. 222° and specific rotation in acetone of +312°.

The striking feature of the biological activity of griseofulvin is its profound influence on the morphogenesis of many fungi. At concentrations of 0.1 to 10 µg./ml. it produces severe stunting and distortion of the hyphae, to which it imposes a spiral twist. Brian (7), from an examination of its effects on many microorganisms, noted that only those fungi with chitin cell walls are sensitive to griseofulvin; fungi with non-chitinous cell walls, actinomycetes, and bacteria are unaffected. The seeds of angiosperms suffered retardation of germination on agar containing 25 µg./ml.

Dechlorogriseofulvin, in which the chlorine of griseofulvin is replaced by hydrogen, was recovered by MacMillan (50) from culture filtrates of both *P. janczewskii* and *P. griseofulvin*. He showed it to be also capable of inducing a twisted growth in *B. allii* although at higher concentrations. Grove and his colleagues (38) suggested that the curling activity may be associated with the spiro structure of the molecule. The practical use of griseofulvin has been limited by poor yields and difficulty associated with its low solubility in most solvents. Ashton and Rhodes (1) pointed out that the first difficulty is overcome by submerged fermentation, and that *N,N'*-dimethylformamide is a useful solvent, particularly for the purpose of formulations for practical use.

As a result of these developments, griseofulvin formulations are, at the time of writing, undergoing field trial as fungicides. The ability of griseofulvin to travel in the transpiration stream of the host plant, demonstrated by Brian and his colleagues (19), may render the compound of great practical interest for the control of those fungus diseases not readily assailable by the standard protective fungicides.

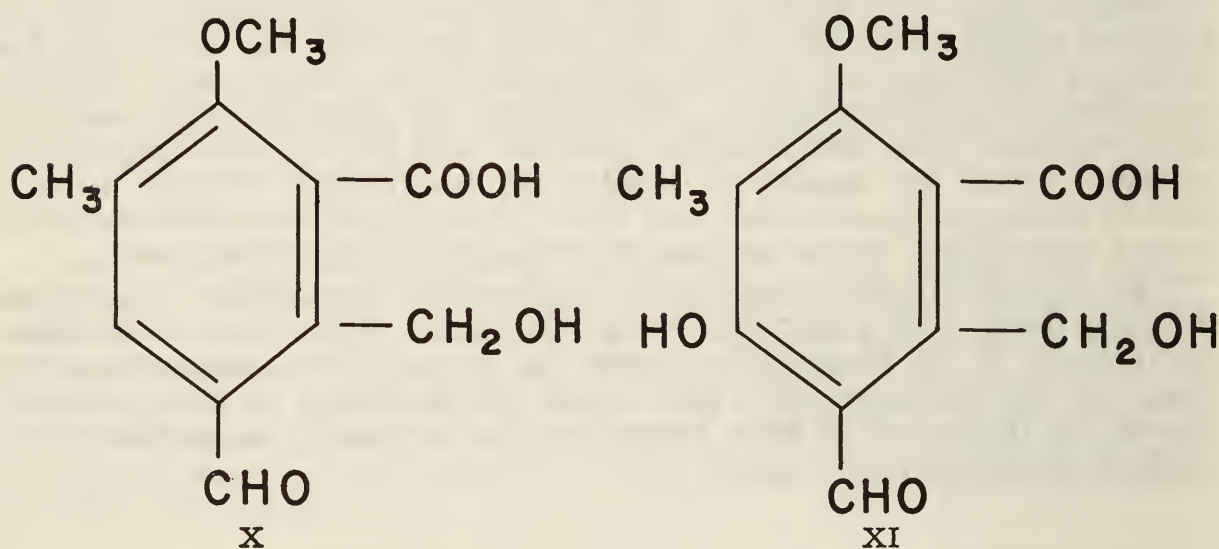


Gladiolic Acid:

In 1946, Brian, Curtis, Grove, Hemming, and McGowan (12) reported that the culture media of *Penicillium gladioli* McCull. & Thom, previously reported to contain powerful bactericides, also had strong antifungal properties. The cultural conditions favoring the formation of this antifungal compound,

which they named gladiolic acid, were studied by Brian, Curtis, and Hemming (13), who showed that the acid was active only in its undissociated form, namely, at a low pH. It forms long colorless crystals of m.p. 160° , is optically inactive, is a monobasic acid formula of $C_{11}H_{10}O_5$, and appears to be a methoxymethyl-2-carboxyphenyl glyoxal. Grove (36) found that treatment with alkali transformed gladiolic acid to an isomer of m.p. 234° and, on the basis of this and other reactions, deduced that gladiolic acid may be represented by the tautomeric keto form VIII (5,6-diformyl-2-methoxy-3-methylbenzoic acid) and the lactol form IX.

From cultures of another strain of *P. gladioli*, Raistrick and Ross (60) isolated a compound which they showed to be dihydrogladiolic acid of probable structure X (5-formyl-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid), for on oxidation it was converted to gladiolic acid.



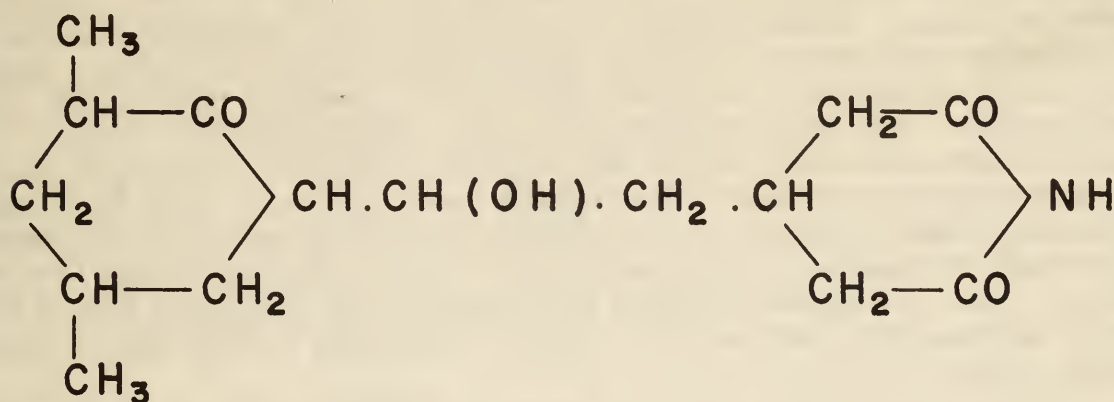
The synthesis confirming the proposed structure of the gladiolic acid was reported by Brown and Newbold (20).

In no case reported by Grove (37) is the fungistatic action of gladiolic acid greater than that of *o*-phthalaldehyde, but it is more specific—a property thought to be due to the presence of the carboxyl group adjacent to the two formyl groups. Raistrick and Ross (60) showed that cyclopolic acid, a metabolic product of *P. cyclopium* Westling, is 4-hydroxydihydrogladiolic acid XI.

Cycloheximide:

Leach, Ford, and Whiffen (48) reported that cultures of *Streptomyces griseus* (Krausky) Waks. & Schatz contained, in addition to the bactericide streptomycin, a toxin active against many yeasts but not against bacteria. As the compound is produced by an actinomycete and was thought to be a diketone, it was originally named actidione. The trade name, "Acti-dione" is registered by the Upjohn Co. for its brand of this substance which is now known as cycloheximide. The chemical studies reported by Ford and Leach

(30) gave the formula $C_{15}H_{23}NO_4$, and degradation studies by Cornfeld, James, and Parke (47) led to the suggestion that the active compound was β -[2-(3,5-dimethyl-2-oxycyclohexyl)-2-hydroxyethyl]-glutarimide (XII) a monoketone.



XII

The colorless crystals of cycloheximide, m.p. $119.5\text{--}121^\circ$, are slightly soluble in water and, though stable in neutral or acid solution, are rapidly decomposed by alkali. All the nitrogen is liberated as ammonia on boiling with sodium hydroxide solution.

Actidione exhibits a marked specificity in its biological reactions. It is, for instance, highly toxic to rats; Ford & Leach (30) place the intravenous LD_{50} at 2.5 mg./kg., whereas that for mice is 150 mg./kg. Similarly, to certain pathogenic fungi it is sufficiently toxic to be suggested for practical use; for example, against powdery mildews (29) and cherry leaf spot (58). Formulated products are now on the market, but its wide use is curtailed by high phytotoxicity.

Trichothecin:

In 1934 Koch (46) reported that, around July and August, the black knots produced on plum and cherries by the fungus *Dibotryon morbosum* (Schw.) T. & S. yield the fungus *Trichothecium roseum* Link, which actively parasitizes the stroma of the pathogen. An example of parasitism by this fungus on a non-pathogenic fungus was described as early as 1909 by Whetzel (81). Culture filtrates of *T. roseum* were found by Brian and Hemming (17) to inhibit the germination of conidia of *Botrytis allii*. The antifungal compound responsible was isolated by Freeman and Morrison (31) and named trichothecin. It crystallizes from light petroleum in slender colorless crystals of m.p. 118° , is readily soluble in organic solvents, but is of only slight solubility in water. It is optically active $[\alpha]^{18}_D +44^\circ\text{C.}$ in chloroform. Trichothecin was shown by Freeman and Gill (32) to be an ester ($C_{19}H_{24}O_5$) of isocrotonic acid and a ketonic alcohol trichothecolone (m.p. 182°), the structure of which does not yet appear to have been determined. Trichothecin is relatively stable under acid and mildly alkali conditions. It is strongly antifungal, inhibiting spore germination of *Penicillium digitatum* Sacc. at $1.25\text{ }\mu\text{g./ml.}$ and of *B. allii* at $6\text{ }\mu\text{g./ml.}$, but is without noteworthy bactericidal activity. It is but partly responsible for the inhibitory effects of culture filtrates of *T. roseum* on plant viruses, a property more pronounced in a polysaccharide fraction examined by Bawden and Freeman (3).

The Ecological and Practical Significance of Fungal Antagonism:

The discussion of the part played in nature by the antifungal toxins produced by soil fungi under the unnatural conditions of a culture medium was, because of the absence of experimental data, initially based largely on *a priori* reasoning. Strong doubts that the toxins have ecological significance arose, for toxin production is usually greatly influenced by the nutrient supply and was found only in media of high nutrient content, which have little resemblance to the natural substrate of the toxin-producing fungus. The search for the antibiotic in normal soil inoculated with the appropriate micro-organism was unsuccessful. This failure may have been due to a rapid adsorption of the antibiotic on soil colloidal matter. Streptomycin, for example, is basic in nature and behaves as if it were a trivalent ion of low zeta potential. For this reason Simonoff and Gottlieb (68) concluded that it is so rapidly inactivated by clays and soil organic matter that it could play no active biological role.

Trichothecin was also found by Hessayon (39) to be strongly absorbed by soil yet, even in unsterilized soil in which *T. roseum* had been grown, he found evidence of toxin production in a modification of the growth of *Fusarium oxysporum* var. *cubense* E. F. Smith on nutrient agar in which the soil had been sandwiched. At sub-lethal concentrations trichothecin, like many other toxicants, is stimulatory: Hessayon observed a stimulation of mycelial growth of *F. oxysporum*, whereas in the sterilized soil infected with *T. roseum* the growth of *F. oxysporum* was inhibited.

Chloramphenicol (chloromycetin), being a neutral molecule, is not inactivated by soil colloids, and Gottlieb and Siminoff (34) detected its presence in normal soil inoculated with *Streptomyces venezuelae*. But this antibiotic is so quickly metabolized by other soil microorganisms that its accumulation in soil can, at best, be only negligible.

Adsorption and the instability of most known antifungal metabolic products of fungi were therefore two of the reasons for the view that these products exerted little ecological influence. In a critical examination of these factors, Jefferys (40) used ten different antibiotics and concluded that, though he produced no direct evidence that any were produced naturally in soil, at least it could be said that all ten showed a sufficient degree of stability to exert a significant biological effect.

A third reason arose through the frequent failure to detect the formation of antibiotics in normal soil. Wright (82), for example, was unable to detect gliotoxin production in normal soil inoculated with *T. viride* and receiving no supplement or heat treatment; but in autoclaved soil gliotoxin was present. Similarly, Grossbard (35), was unable to detect the presence of antibiotics in non-sterile soil inoculated with *Penicillium patulum* Bainier, with *Aspergillus terreus* Thom, or with *A. clavatus* Desm. The complication due to the liberation of nutrient in most soil sterilization procedures was reduced by Grossbard who used propylene oxide as sterilant. She was able to detect antibiotic production in soil so sterilized before inoculations, especially when glucose or other carbohydrate nutrients were supplied. Success in the long search for antibiotics in untreated soil was met by Wright (83) who found gliotoxin in the seed coats of peas planted in a potting compost—the first example of antibiotic production in an uninoculated soil in the vicinity of a growing plant.

Grossbard found the type of organic material used as a source of carbohydrates had a marked effect on antibiotic production. Certain samples of red clover or rye grass were unsuitable—an observation recalling the work of West and Hildebrand (80), who found that the plowing of soybean cover

crop into soil infected with strawberry root rots produced a striking reduction in the incidence of the disease, whereas the plowing in of red clover had little effect. Similarly, Rouatt and Atkinson (65) found that the plowing of soybean into potato scab-infested soil led to a reduction of scab incidence, an effect not produced by plowing in red clover or rye grass. The significance of the increased acidity in the soil manured with soybean should not be overlooked. Grossbard concluded that the modification of the soil microflora produced by green manuring resulted in the formation of local concentrations of antibiotics, perhaps only temporary, which may have exerted an inhibitory action on plant pathogens in the neighborhood of the roots of host plants. A similar conclusion was reached by Stevenson (69), who obtained an apparent correlation between the control of root rot of wheat in soil inoculated, after sterilization, with various actinomycetes and the inhibition of the causal fungus *Helminthosporium sativum* by the actinomycetes in nutrient culture.

The appearance of antibiotics in sterilized soil inoculated with the appropriate fungus may be influenced not only by the improved nutrient but by the absence of other microorganisms. Wright (82) tested this possibility by adding a mixed microflora to autoclaved soil, but concluded that the beneficial effect of autoclaving was not merely a consequence of sterilization, for whereas 5 min. steaming eliminated the microflora, a minimum of 25 min. steaming was necessary to make the soil a suitable medium for gliotoxin production. Nevertheless, the elimination of competing organisms cannot help but encourage growth of the antibiotic-producing fungus. This outcome was demonstrated by Richardson (64), who showed that treatment of the soil with the dithiocarbamate fungicides, thiram and ferbam, reduced the number of fungal species, the survivors being predominantly species of *Trichoderma* and *Penicillium*, which tolerate the fungicides. Protection of pea seedlings from damping-off due to attack by *Pythium ultimum* Trow was achieved in the thiram-treated soil for periods long after the content of thiram had fallen below levels toxic to the pathogen, especially in compost-rich potting soil. Although no decisive test was made of whether the suppression of the pathogen was due to competition by resistant saprophytic fungi or to direct antagonism, the knowledge that the latter could produce antibiotics adds weight to the concept that the effectiveness of the fungicide is here due to antibiotics produced by the fungicide-resistant soil fungi.

An even more conclusive example is provided by the work of Bliss (4) on the role of fungicidal fumigants in the control of the honey-fungus, *Armillaria mellea* (Vahl.) Quel. The thick-walled cells, the pseudosclerotia, which form particularly on the rhizomorphs of this fungus, protect it not only from fumigant but from the attack of *Trichoderma viride*, which Bliss found was constantly associated with infected roots after fumigation. He concluded that the disturbance of the microbial soil population which follows fumigant treatment permits a rapid development of *T. viride*, which is now able to overcome the protective mechanism of *Armillaria*. The control of *Armillaria* in citrus soils by fumigation is, he considered, due primarily not to the fumigant itself but to the antibiotic action of *Trichoderma* made possible by the effects of the fumigant on the microbial population of the soil.

Further evidence of the ecological significance of fungal toxins was provided by studies on mycorrhizal relationships. The failure referred to on p. 56, of attempts at afforestation in certain sandy heath soils was found by Rayner (61, 62) to be rectified by the addition of compost at the time of planting. Having shown that the failure was not due to nutrient factors, she suspected that the effect was associated with biological activity brought about by the supply of organic matter. Her husband, Neilson-Jones (55), demonstrated that the reason for the inhibition of conifer growth in the affected soil

was the presence of antifungal compounds to which at least one mycorrhizal fungus, *Boletus bovinus*, was highly susceptible. Brian, Hemming, and McGowan (16) examined the fungi present in this heath soil and found only certain species of *Penicillium* which produced gliotoxin in liquid culture. Jefferys, Brian, Hemming, and Lowe (41) confirmed antibiotic production by microfungi of acid heath soils and concluded that the factor is of ecological significance. If this is so, the reason for the ameliorative action of compost remains to be explained; the encouragement of the growth of microorganisms at the expense of the antibiotic-producing fungi, or the adsorption of the antibiotics produced, are two possible hypotheses.

A somewhat parallel phenomenon was found by Melin (51) in Swedish forests where he demonstrated that needles shed from the trees contained water-soluble and heat-stable substances having a strong inhibitory effect on mycorrhizal growth, but with a beneficial or negligible effect on litter-decomposing species of fungi. The toxic substance was not identified.

The Practical Utilization of Fungicidal Antibiotics:

Although it is probable that the success of cultural practices such as green manuring is due to the encouragement of the growth of antibiotic-producing fungi, and that these practices can be aptly classed as methods of biological control, a more direct use of the isolated fungicidal antibiotic merits exploration. In addition to the extensive tests, in recent years, of the field performance of formulations of cycloheximide and of griseofulvin mentioned in the relevant sections above, a number of other antibiotics have received attention. Those that show the ability to impart fungicidal properties to the treated plants are the natural first choice for such tests. This property, in the case of griseofulvin, was demonstrated by Brian *et al.* (19) and by Stokes (70) by the ingenious technique of the examination of the guttation drops from oats and wheat grown in water culture containing the antibiotic. Crowdy and Pramer (22), by the same method, found that chloramphenicol and penicillin were translocatable, whereas streptomycin, though not present in the guttation drops, was detected in the leaves of the treated plants. Of the actinomycete antibiotics, chloramphenicol and streptomycin were identified in the leaf tissue, but no clear evidence was obtained of the uptake and translocation of chlortetracycline, neomycin or oxytetracycline (to use their non-proprietary names).

Another potential use of antibiotics for the purpose of crop protection is in seed treatment, an early example being the use, by Wallen *et al.* (74), of cycloheximide for the treatment of pea seed. Internal seed disinfection of peas infected with *Aschochyta pisi* Lib. was reported by Dekker (23), who used as seed disinfectant a brei of culture of *Streptomyces rimosus*. The effective fungicide was found to be rimocidin, treatment of the pea seed with a crude preparation of this compound gave disease-free seedlings. Rimocidin is a polyene which was considered by Oroshnik and his co-workers (56) to be a tetraene, i.e., containing the group $-(CH:CH-)_4$. It is convenient here to refer also to the successful control of barley smuts *Ustilago hordei* (Pers.) K. and S. and *U. nuda* (Jens.) Rostr. obtained by Chinn and Russell (21) by soaking the infected seed in broth cultures of the bacterium *Pseudomonas viscosa* (Frankl. and Frankl.) Migula. These authors at first considered that this effect was due to metabolic substances produced by *P. viscosa*, but mentioned that the results of more recent experiments suggested that microorganisms play little or no part in the control.

A discussion of the chemistry and biological properties of other antibiotics that are of potential fungicidal use would go beyond the field of this review, for there is no evidence that many of them are derived from plant pathogens or that they function in nature as ecological chemicals. It will suffice to say that streptomycin, oxytetracycline, cycloheximide and other antibiotics, are available as formulated products for use as agricultural fungicides. Their use, and tests of other antibiotics not yet commercially available, have been reviewed recently by Brian (9) and by Ark and Alcorn (2).

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Chapter 9

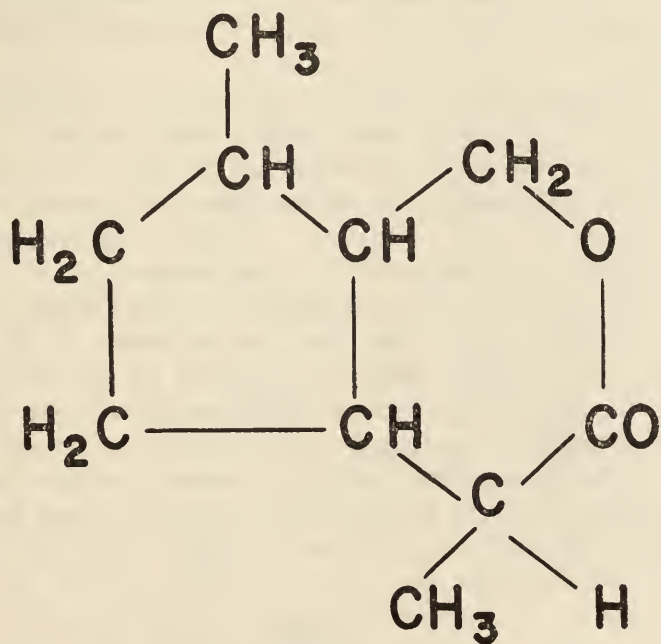
THE PRODUCTION OF INSECTICIDES AND INSECT ATTRACTANTS BY INSECTS AND MICROORGANISMS

Known examples of the production of insecticides by insects are few and recent. The chemotropic attraction of males by females of certain moth species has received more detailed study; early naturalists were amazed at the remarkable distances over which these males are guided to the waiting females. It is convenient to include in this discussion on insect-produced insecticides a recent example of a bacterial insecticide. Moreover, there are one or two known cases of the production of biologically active chemicals by organisms outside the categories of the chapter headings, which may be included in the present chapter.

Of the insecticides for discussion, the first is:

Iridomyrmecin:

The Argentine ant (*Iridomyrmex humilis* Mayr.) and other ant species characterized by anal glands produce an insecticidal agent, iridomyrmecin, and it is reported in B.P. 715,546 that up to 3.5 g. may be recovered in the summer season from a million unfed worker ants. The insecticide is of low mammalian toxicity, its formula is $C_{10}H_{16}O_2$, and it is an optically active lactone of a cycloparaffin which, according to Fusco, Trave, and Vercellone (15), on oxidation yields a dicarboxylic acid identical to nepetalinic acid. This discovery, confirmed by Cavill, Ford, and Locksley (11), indicates that iridomyrmecin is of structure XIII, a structure capable of epimerization of the asymmetric carbon adjacent to the carbonyl group.



XIII

***Habrobracon* Venom:**

Beard (9) drew attention to the paucity of information on the toxicology of the poisons by which many braconid wasps paralyze the insect larvae upon which they feed and on which they deposit eggs. He chose for study *Habrobracon juglandis* Ashmead, which attacks the larvae of such insects as *Ephestia*, *Plodia*, and *Galleria*. The paralysis of the attacked larvae is due to the injection of a venom, which can be collected from the stylets of the wasp, and which is of extreme potency. Beard calculated that a single wasp could produce enough venom to kill about 50 pounds of *Galleria* larvae. The venom is, to some degree, species-specific though the wasp limits its attack to relatively few species. It will not attack larvae of the Japanese beetle (*Popillia japonica*) or the European corn borer (*Pyrausta nubilalis* Hb.), nor did injection of the venom into larvae of these species produce paralysis. It is of interest in connection with the previous discussion of the Hopkins Host Selection Principle (see p. 22) that the wasp, if presented a mixed batch of *Ephestia* and *Galleria* larvae, will first attack *Ephestia*, even though it had been reared for several generations on *Galleria* larvae.

The venom is inactivated at temperatures of 65°C. or higher, and is presumably protein in character. Its mode of action is unknown, but is different from that of the more common insecticides of the organophosphate and DDT series. The protein nature of the venom prompted Beard to suggest that it is an enzyme, though he reported no attempt to determine whether it had any relationship to the lecithinases and cholinesterases of the snake venoms.

The Insecticidal Inclusions of Bacillus cereus:

A number of bacterial species, which can be classified as variants of *B. cereus* Frankland & Frankland, are pathogenic to insects and have indeed been employed as agents in biological control: e.g., *B. thuringiensis* Berliner against the alfalfa caterpillar *Colias philodice eurytheme* Boisduval (28), and strains of *B. cereus* against codling moth (*Carpocapsa pomonella* L.) (30). *B. sotto* Ishiwata and *B. cereus* var. *alesti* Toumanoff and Vago (32) are pathogens of the silkworm *Bombyx mori* L.

Hannay (19) observed that, in the sporulation of *B. thuringiensis*, the spores are invariably accompanied by a parasporal body, a highly refractile and well-formed diamond-shaped crystal. He found that most other variants of *B. cereus* pathogenic to insects formed crystals on sporulation, whereas non-pathogenic strains failed to form crystals. Hence, he suggested that crystal formation is in some way connected with the formation of a toxic substance causing septicaemia in insect larvae. Hannay's observation was confirmed by Steinhaus and Jerrel (29) within the range of strains of *B. cereus* available in their laboratory. The crystal material proved to be protein in character and readily dispersible in alkali, both properties known to be shared by the polyhedral bodies that appear in certain virus-infected insects. It was therefore important to ascertain whether similar viruses were involved, but electron micrographs revealed no trace of such particles (21). That the crystal protein is the toxin was shown by Angus (5), for the spore-free alkaline extract of a culture of *B. sotto* fed to silkworm larvae rapidly caused paralysis, whereas the sediment of washed alkali-treated spores had no effect when fed but produced septicaemia when injected into the larvae (7). The potency of the crystal toxin is such that the LD₅₀ is less than 0.5 µg. per g. larva—about two hundred times as toxic to silkworm larvae as the toxin derived from *Corynebacterium diphtheriae* (Flügge) Lehmann & Naumann.

Methods for the recovery of the material of the crystal from the sporulating spores are described by Hannay and Fitz-James (21). Their chemical studies revealed that the crystal is wholly protein, containing no phosphorus and composed of at least seventeen amino acids, all α -amino acids commonly found in proteins. It would appear, from the preliminary results of Angus (6), that only those insects of high gut pH are susceptible to either *B. sotto* or its toxin; but as Hannay (20) pointed out, this effect may be more closely associated with enzyme activity than with pH. He speculated that septicemia, as distinct from paralysis, is an outcome of an invasion in larval tissues by bacteria present in the gut.

Insecticidal Emanations from Insects:

Gough (16), in studying the resistance of the flour moth *Tribolium confusum* Duv. to hydrogen cyanide, observed that in certain circumstances the adult beetles, and particularly the virgin females 4-5 weeks old, emitted a substance which he described as self-toxic, for it killed non-emanation-producing beetles enclosed in the same container. He did not examine this insecticide chemically beyond recording that a flake of potassium hydroxide suspended over the beetles turned green, and that the emanation affects HCN mortality in a manner that suggests that it forms a loose combination with HCN, removing it from the gas phase to the walls of the container.

It had long been known (see Chittenden, 12) that flour heavily contaminated with *Tribolium* became pink in color, and Roth and Howland (25) collected, in a dry-ice trap, yellow-brown crystals from air drawn over adult *T. confusum*. The material collected in a similar manner from adults of *T. castaneum* Hbst. was shown by Alexander and Barton (4) to be a mixture of 2-ethyl-1,4-dihydroxybenzene and 2-methyl-1,4-dihydroxybenzene. Their results were confirmed by Loconti and Roth (26) who, on spectrographic evidence, concluded that these quinones were also produced by the adults of *T. destructor* Uyttenb. and of the long-headed flour beetle *Latheticus oryzae* Waterh. Loconti and Roth sought the function of these secretions and found that their presence had little effect on population growth but that they served rather as repellents, for starved beetles showed a marked preference for fresh flour over that contaminated with the quinones.

A similar example of auto-intoxication was described by Roth, Neigisch and Stahl (26) in the cockroach *Eurycotis floridana* Walker, but in this case the secretion was identified as *trans*-2-hexenal which, though toxic to the roaches kept in a closed space and made to emit the material, seems normally to function as a repellent.

The release of hydrogen cyanide by certain millipedes was described as early as 1882 by Guldensteeden-Egeling (17). Davenport and his colleagues (13) noticed a cyanide smell when collecting the luminous millipede *Lumino-desmus sequoiae* Loomis & Davenport, and established that it was due to hydrogen cyanide. Bees imprisoned over the millipedes for 15 minutes were killed. The ability of the millipedes to generate hydrogen cyanide may be associated with the luminescence, for McElroy and Strehler (23) showed that cyanide stimulated light-emitting reactions in other organisms, but Davenport *et al.* could trace no tangible connection between cyanide release and luminosity.

Insect Attractants Produced by Insects:

Benzene extracts of the abdominal tips clipped from the virgin gypsy moth *Lymantria dispar* L. will attract males of the same species. Haller, Acree and Potts (18) showed that the attractant, which Acree (1, 2, 3) named gyptol,

is an alcohol occurring in the phthalic acid ester fractions isolated from the unsaponifiable material of the benzene extract. Acree used chromatographic methods for the concentration of the alcohol as the succinic acid ester or as the azoate (*p*-phenylazobenzoyl ester), but has as yet not been able to define more closely the attractant compounds. In his later chromatographic studies Acree (3) recovered three active esters including gyptol, which appears to be esterified in the natural attractant; the three esters are derived from at least two different alcohols.

Even the housefly (*Musca domestica* L.) is more attracted to poison bait which other flies have previously visited than it is to fresh baits; the factor responsible was extracted, by Barnhardt and Chadwick (8), from flies with 95 per cent ethanol. Similarly, Dethier (14) found that sugar baits became more attractive to blowflies through the agency of the flies themselves, and concluded that the attractant was a volatile compound produced from the sugar by some reaction of the flies.

Acrasins:

A remarkable example of chemotropism is provided by certain slime molds which secrete substances causing the aggregation of the individual cells to form organized communities. The life history of *Dictyostelium discoideum*, for instance, begins with the discharge from the spores of an amoeba-like cell which feeds by phagocytosis and reproduces by binary division. After this vegetative stage the myxamoebae begin to collect in large aggregates, which assume a rounded structure with a hollow neck or stalk. Some cells become large and evacuolated, others appear to climb to the stalk, where they eventually form what might be called spores.

This process was first described in 1902 by Olive (24), and in 1947 Bonner (10) showed that the aggregation stage is a chemotropic response to a substance, which he named acrasin, secreted by the myxamoebae. Investigations of its chemistry have been frustrated by its apparent instability though Shaffer (27) found it to be stable if quickly frozen. In a recent article he (27a) gives evidence that acrasin is accompanied by a protein—probably an enzyme—which inactivates it and that, when freed by dialysis, the acrasin is remarkably stable.

It would appear that a number of acrasins exist. For example, Shaffer (27) found that young cells of the slime mold *Polysphondylium* produced an acrasin to which cells of *Dictyostelium* did not respond; acrasin secreted by older cells caused aggregation of cells of both species. Sussman (31) considered that the initiation of aggregation should be distinguished from the continuance of aggregation. It seems reasonable to assume that the chemotropic agents concerned in the aggregation of the slime molds represent but slight modifications of a basic acrasin molecule.

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Chapter 10

BACTERIA vs. HIGHER PLANTS

In closing the discussion on the fungus-host plant relationships, attention was drawn to the resemblances between the symptoms of the diseased plants and those produced by the application of growth substances. This similarity is also shown among bacterial diseases of plants, e.g., crown gall disease.

The crown gall problem has been reviewed in detail by de Ropp (9) and by Klein and Link (12). The causal organism, *Bacterium tumefaciens* (Smith & Town.) Conn., was shown by Berthelot and Amoureaux (1) to be capable of producing indoleacetic acid from nutrient media containing tryptophan. Attenuated strains of *B. tumefaciens*, unable of themselves to cause gall formation, can do so if indoleacetic acid or a related growth substance is supplied. This observation of Braun and Laskaris (6) and of Thomas and Riker (14) led directly to the conclusion that the difference between attenuated and virulent strains of the bacterium is in the relative amount of growth substance produced by each. The remarkable feature of the crown gall disease is, however, that host tissue, once transformed to gall tissue, continues to produce growth substance in greater than normal amounts, even in the absence of disease-causing bacteria. This bacteria-free gall tissue, when implanted into healthy tissue of the same host species, was found by Braun and White (7) to cause the development of typical crown galls. It is therefore necessary to postulate, in addition to indoleacetic acid which is the factor responsible for the continued abnormal proliferation of the gall tissue, a second factor which Braun has termed the "tumor-inducing principle", and which renders the gall tissue capable of producing the growth substance in the absence of the pathogen. This hypothesis is needed to explain the fundamental difference between the abnormal growth induced by indoleacetic acid and that caused by crown gall tissue; the former growth survives but briefly; the latter is a permanent and continuing alteration of the growth pattern.

Braun's "tumor-inducing principle" has not been isolated but its study is possible in tissue culture. The possibility that this principle is a virus is not supported by the experimental work reviewed by Braun (4), nor has the electron microscope revealed evidence of virus particles. The "tumor-inducing principle" is thermolabile and of high molecular weight; its precipitation by protamine sulphate and by manganous chloride indicates that it is a nucleic acid rather than a protein. Its remarkable ability, discovered by Klein and Klein (11), of conferring the heritable property of virulence to avirulent strains of *B. tumefaciens*, *Agrobacterium radiobacter*, *A. rubi*, and *Rhizobium leguminosarum* brings the "tumor-inducing principle" into the broad category of transformation agents; these will be further discussed on page 84.

The establishment of symbiosis between *Rhizobium* and its host legume is another example of an interaction between a bacterium and its host plant which involves auxins. The first observable stage is a characteristic deformation of the root hairs of the legume, which was thought by Thimann (13) and by Chen (8) to be due to the secretion by *Rhizobia* of indoleacetic acid. Chen demonstrated the production of this compound by *Rhizobia* growing in culture media containing tryptophan; Thornton and Nicol (15) produced root-hair curling by treatment with sterile filtrates from *Rhizobia* cultures. This

the lactone ring being formed between the hydroxyl group of the lactic acid residue and α -carbonyl group of tabtoxinine, an amino acid (α , ϵ -diamino- β -hydroxypimelic acid) not previously isolated from natural sources. The lactone ring is opened by alkaline hydrolysis, and the resulting compound is not phytotoxic as is tabtaxinine, so that it would seem that ionizable groups at both ends of the molecule render it inactive. The active products (XV) and (XVI), and also methionine (XIV), have but one ionizable end group; but Braun (5) found that an additional and uncharacterized end group is necessary, for none of the other methionine-like compounds that he tested was phytotoxic.

P. tabaci when carried in culture for long periods may lose its ability to produce the toxin. Braun (2) was unable to distinguish the non-toxigenic strains from the milder pathogen *P. angulata* (Fromme and Murray) Holland. This example provides an introduction to the subject of bacterial dissociation which will be discussed on page 79.

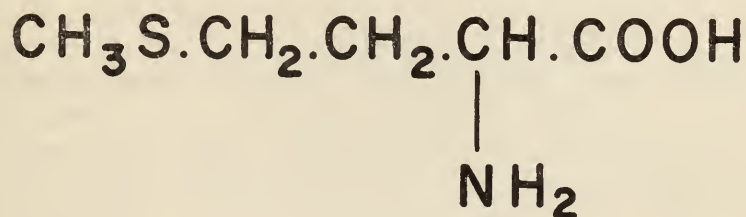
It may be anticipated that further studies of the phytopathogenic bacteria will produce further examples of phytotoxin production by these organisms. Certainly a wide range of toxins are known to be involved in the bacterial diseases of man and of animals, and as far back as 1936 Topley and Wilson (16) wrote, "The basis of all harmful effects of bacterial infection is quite certainly chemical; and only when the chemist has replaced the immunologist shall we be able to give an intellectually satisfying account of what happens when a particular parasite invades a particular host". This statement was retained by Wilson and Miles, on p. 1146 of the 4th edition of this masterpiece, published as recently as 1955.

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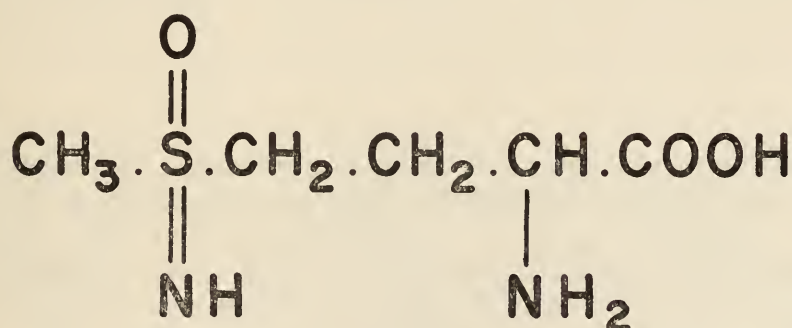
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reaction is produced even by strains of *Rhizobium* incapable of establishing infection of the rootlets. No explanation is as yet available of the remarkable specificity shown by *Rhizobia* in their choice of host legume.

An example of phytotoxin production by a pathogenic bacterium is provided by the leaf spot disease of tobacco produced by *Pseudomonas tabaci* (Wolf and Foster) Stevens, a disease so rapid in development and so devastating to foliage that it is called "wild fire". Johnson and Murwin (10) found that sterile filtrates of cultures of *P. tabaci* could produce the symptoms of "wild fire" not only in tobacco, but in many other plant species. Because the lesions produced by the filtrate are indistinguishable from those produced by infection it seems safe to conclude that the toxin is produced by the pathogen. The observation of Braun (3) that the toxin inhibits the growth of *Chlorella*, an inhibition prevented by the addition of yeast or liver extract, led him to test the effects of the vitamins known to be present in such extracts. Of these growth factors, only methionine (XIV) in its natural (L-) form inactivated the toxin. Moreover, he showed that chlorotic lesions, similar to those of "wild fire" disease, are produced by treatment of tobacco with methionine sulfoximine (XV), then recently shown to be responsible for the convulsive effect on dogs of wheat flour treated with nitrogen trichloride.

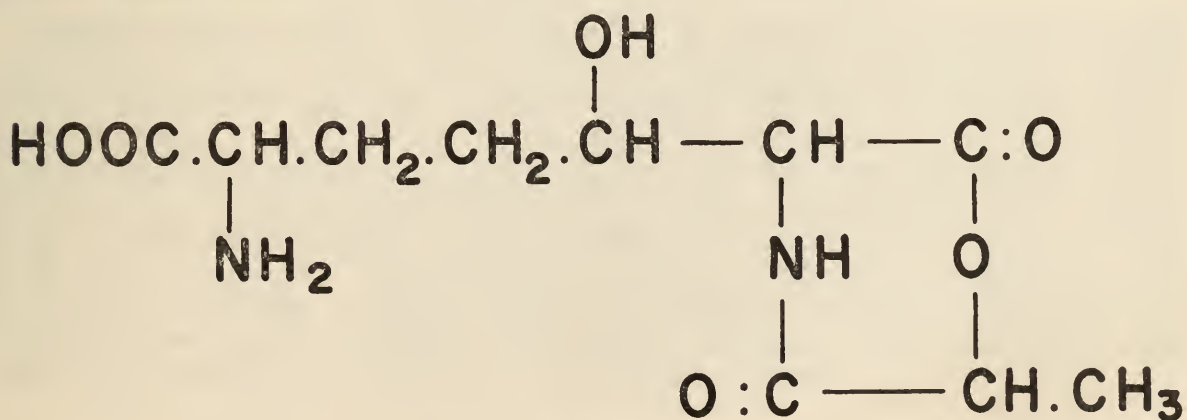


XIV



XV

This valuable clue enabled Woolley, Schaffner, and Braun (17) to deduce that "wild-fire" toxin is probably the lactone of α -lactylamino- β -hydroxy- ϵ -aminopimelic acid (XVI),



XVI

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Chapter 11

THE ECOLOGICAL CHEMISTRY OF BACTERIA

Whereas the toxins produced by fungi are, as described in Chapter 7, compounds of diverse structure not easily falling into any of the broad groups of metabolic products, the antibiotics produced by bacteria are, by and large, polypeptides. Indeed, many authorities, for example Van Heyningen (56), included the requirement of antigenicity in the definition of a bacterial toxin. Fluvomycin and the bacitracins, for instance, are polypeptides; subtilin and nisin are polypeptides containing an unusual amino acid β -methyl lanthionine (α -amino- β -(2-amino-2-carboxyethyl-mercapto)-butyric acid) (2, 44). The tyrocidines and gramicidins are cyclic polypeptides and have provided excellent material for the elucidation of the sequence of the amino acids composing these compounds, a brilliant example being the unravelling of the structure of tyrocidine A by Paladini and Craig (45).

But, as yet, the significance of these bacterial products in the interplay of the soil microflora or in the maintenance of plant health has not been exposed, nor have the known bacterial toxins and antibiotics found use in crop protection. However, there is a growing literature dealing with the effects of environmental chemicals upon the behavior and properties of bacteria. A discussion of these effects, which will inevitably involve the viruses, may be opened with a description of the bacteriocins, for these compounds are nearest to the bacterial antibiotics.

Bacteriocins:

In 1925 Gratia (24) reported the production, by strains of *Escherichia coli* (Migula) Castell. & Chalmers, of readily dialysable compounds of high toxicity to certain other strains of this bacterium. These compounds were termed colicines by Fredericq and Gratia (21) but, because like compounds have been since isolated from cultures of *Shigella dysenteriae* (Shiga) Castell. & Chalmers and less commonly from *Salmonella*, the more general name of bacteriocins is preferable (30). Their production is not confined to enterobacterial species, for Jacob (29) has recently reported the isolation from cultures of *Pseudomonas pyocyanae* (= *Ps. aeruginosa* (Schroeter) Migula) of pyocine, which has the typical properties of a bacteriocin.

These compounds are non-antigenic but, as shown by Heatley and Florey (26) and by Gardner (23), are proteins or polypeptides susceptible to hydrolysis by proteolytic enzymes. Their biological activity is extremely specific for, as a general rule, the colicine produced by one strain of *E. coli* is toxic only to sensitive bacteria of the same strain. But the most remarkable feature of the group, a phenomenon discovered by Jacob, Simonovitch, and Wollman (31), is that colicine synthesis can be induced in cultures of certain strains of *E. coli* by treatment with ultraviolet light or with certain chemicals such as nitrogen mustards. Colicine cannot be detected in such cultures prior to irradiation, but after treatment the bacteria continue to grow until the cells lyse, when free colicine appears in the culture media.

The ability of a particular bacterial strain to produce bacteriocins is heritable — strains having this ability are said to be bacteriocinogenic. But the power, when induced, is lethal to both bacteriocinogenic and normal strains of the bacteria.

Bacteriocinogenicity and its consequences have parallels in the phenomenon of lysogeny. Fortunately, this involved subject has been brilliantly reviewed by Lwoff (37).

In brief, lysogeny is the phenomenon by which certain bacterial strains have a heritable ability to produce bacteriophage. The cells of such lysogenic strains may spontaneously lyse, with the liberation of bacteriophage particles, or the production of the phage may be induced by certain treatments. Such treatments include irradiation by ultraviolet light or exposure to certain chemicals and cause the hitherto latent phage to develop; the affected cells eventually lyse and release phage particles able to attach and multiply in strains of bacteria which are sensitive to the phage.

The analogy between bacteriocinogenicity and lysogeny is strengthened by the similarity in the chemical agents capable of inducing the formation of bacteriocins and bacteriophages; e.g., the nitrogen mustard methyl bis-(β -chloroethyl)amine and tertiary butyl peroxide. These compounds, like ultraviolet radiation, are also mutagenic and carcinogenic.

Lwoff extends the analogy by pointing out that one particle of either colicine or bacteriophage is enough to kill the sensitive bacterium and that the adsorption of either particle by the bacterium is controlled by a specific receptor sometimes common for phage and bacteriocin. In composition, however, bacteriocins are wholly protein, whereas the bacteriophages are organized "bodies" with a protein-lipin membrane containing deoxyribonucleic acids. Presumably the ability of bacteriophage to multiply in sensitive bacteria is attributable to the specific deoxyribonucleic acids; bacteriocins have no such component and do not multiply in the bacteria they attach.

To what then is due the difference between lysogenic and non-lysogenic bacteria? Burnet and McKie (11) concluded that there is some specific thing being perpetuated in lysogenic bacteria, something which Lwoff and Gutmann (38) named the prophage. The prophage has the properties of a genetic unit, incorporated into the genetic material of the bacterium, which can in the terms of Jacob and Wollman (32) "undergo transition into a pathogenic form, the development of which leads to the death of the host and liberation of infective virus particles".

The feature that brings this phenomenon, and also that of bacteriocinogenicity, within our purview is that environmental chemicals can bring about striking changes in cultures of lysogenic and bacteriocinogenic bacteria. In the presence of inducing chemicals such cultures, which otherwise would remain apparently normal, undergo changes which lead to the lethal biosynthesis of bacteriophage or bacteriocin.

A relationship possibly of fundamental significance has been traced by Freeman (22) between lysogeny and toxigenicity in *Corynebacterium diphtheriae*. He found that non-toxigenic strains of this organism became toxigenic after infection with a temperate bacteriophage. The temperate bacteriophage does not kill its host bacterium but renders it lysogenic. Further Freeman found that the production of diphtheria toxin by lysogenic strains of the bacterium is inhibited when iron is present in the culture medium. Mitsuhashi, Kurokawa, and Kojima (39) showed that no appreciable toxin is produced until the exogenous supply of iron of the nutrient has been exhausted. The bearing of this observation on the pathogenesis of diphtheria is discussed by Pappenheimer (46) in connection with the hypothesis that the diphtheria

toxin is related to the protein moiety of diphtheria cytochrome b_1 and that its high toxicity is due to interference in the cytochrome system—a hypothesis supported by the results of Pappenheimer and Williams' study (47) of the insecticidal action of the toxin on the *Cecropia* silkworm.

Although it is hazardous with our present knowledge of the nature of viruses to group together bacteriophages, insect viruses, and plant viruses, on any basis other than that all behave as pathogens of dimensions less than 200 millimicrons, they all consist, chemically, of a greater or lesser complex of protein and nucleic acid. This is the excuse for discussing, in this section, an example of the profound effect that the chemicals of the environment may exert on certain virus diseases of insects. In 1944, Yamafugi and Shirozu (61) found that silkworms fed with hydroxylamine developed a typical polyhedral virus disease, and their claim was confirmed in subsequent publications surveyed by Yamafugi and Yoshihara (62). The effective chemicals included inorganic nitrites and various oximes which were thought by the Japanese workers to lead, by enzymic reactions, to an accumulation of peroxides. Incidentally, Yamafugi and Fujiki (60) also claimed that the treatment of tobacco with heat, hydrogen peroxide, or hydroxylamine leads to the formation of an infectious substance in the leaves, though the degree of its infectiveness was small in comparison with that of tobacco viruses.

Although Yamafugi (59) does not agree, the general view of other virologists is that the Japanese results fall into line with other evidence that the insect larvae before treatment are infected with a latent form of the virus. Smith and Wyckoff (51) recounted several examples of the sudden appearance of virus infection in stocks of caterpillars reared with every care through many generations of apparently healthy stock, and suggested that virus in a latent condition had passed from generation to generation in the stock. Bergold (8), in a complete review of the problem, accepted the latency of insect viruses as a fact—though pleading for further research. He cited the conclusion of Vago (55) that the pathogenic agent is present in most healthy individuals of *Bombyx mori* and can be activated by certain chemicals or physical treatments. If that view is correct, the possibility of latent infection so complicates the study of virus diseases that the use of chemical activation as a means of detecting the presence of latent infection should be more widely explored.

Bacterial Dissociation:

The variability of microorganisms in culture is notorious. The multitude of variants is the despair of every biologist except, perhaps, the systematist; Brierley (10) after half a life's work on the taxonomy of *Botrytis cinerae* with its many strains wrote, "From long, perhaps unduly long, experience of these races I can recognize—"intuitively" if you like—differences which I can neither measure nor describe;"

Typical examples of the changes that appear, and that to become apparent are transmitted from parent to progeny, are the emergence of avirulent strains among pathogens or of strains resistant to toxicants or to virus. Although the expression dissociation, originally used by De Kruif (14) rather in the sense of the mechanical separation of variants from a mixed culture, came to be applied to the appearance of variants of different growth pattern (e.g., rough or smooth) on solid media, it now serves as a term to cover all types of variation and is useful because it implies no mechanism for the changes.

It was at one time thought that the variation in morphological characteristics, frequently found in bacteria, represented different stages in an orderly life cycle—an idea termed cyclogeny by Enderlein (17). This idea proved

inadequate, and it survives possibly only to account for the L-forms of certain bacteria. Under conditions unfavorable to their growth, such as exposure to antibiotics, to antibodies or to phages, bacillary forms of certain species form chromatin-rich granules which develop into tiny "L-phase" colonies on culture media, a phenomenon reviewed by Dienes and Weinburger (16). In a number of cases cited by Kleineberger-Nobel (33), the L-phase has been observed to revert to the bacillary form often with morphological, antigenetic, or biochemical properties different from the original.

Cyclogeny was replaced by the idea that the variants pre-existed in the inoculum and that one or more became predominant because environmental factors happened to favor their development. This hypothesis of pre-existing variants does not, of itself, imply any mechanism for the origin of such variants. This problem, the origin of variant forms, has provoked controversies reminiscent of the earlier battles from which the Lamarkian hypothesis of the inheritance of acquired characteristics emerged discredited. The participants of the controversy tend to two opposing views: at one extreme is the hypothesis that the variants arise by spontaneous and uncontrollable changes in the genes, changes termed mutation by de Vries (15); at the other extreme is the hypothesis that the organisms have a capacity for adaptation to their environment, the adaptive changes being passed on to the descendants.

Although a detailed discussion of this problem, which has been the subject of full symposia in 1953 (1) and 1955 (50), is beyond the scope of this report, the question has so high a potential significance in ecological chemistry that a discussion of the chemical aspects is not out of place.

Experimental evidence that the environment has merely favored the growth of pre-existing mutants and not induced new mutants comes from the fluctuation test of Luria and Delbrück (36) or its modifications. In most cases, a list of which is given by Newcombe (43), such statistical techniques show that the probable explanation of the results is that the mutants were present prior to the exposure of the organisms to the particular selective environment that revealed the mutant. Rates of mutation can be calculated for the characters studied: typically they range from one per thousand to one per thousand million per bacterium per division cycle. In other words there exists, for each gene studied, a small probability that mutation will occur during the "life-time" of the bacterium. Mutation results in the appearance of an altered gene which confers the selected property, e.g., drug resistance, on those bacteria which carry the altered gene. Little is known of the nature of this "accident" presumably in gene duplication.

It was at one time held that this accident in gene duplication which leads to dissociation cannot be an effect of the chemical environment to which the microorganism is exposed. One reason for this view is the evidence of the extreme stability of the gene, as revealed by the constancy of hereditary characters and the high degree of agreement of segregation to the theoretical Mendelian ratios in sexually reproducing organisms. A second reason is that the genes lie within the cell membrane and are well protected from the external environment.

The significance of this second argument was reduced almost to vanishing point by the discovery of many chemicals that are able to increase the rate of mutation. In 1927 Muller (42) showed that mutation rates in the fruit fly *Drosophila* can be increased one or more hundred fold by X-rays. In 1942, Auerbach and Robson (4) found that certain chemical warfare agents, such as the mustard gas, β,β -dichloroethyl sulphide, increased mutation rate in the same insect. Since that date, the number of chemicals with mutagenic properties has grown to proportions beyond listing here. Levan (35), for instance,

gave a list with references of over twenty-five different types of compounds capable of inducing chromosome changes in *Allium* and *Vicia*. Even the mutagenic action of irradiation is biochemical, for Stone, Wyss, and Haas (54) found that the irradiation of the medium prior to inoculation with the bacteria will increase mutation rates in the latter. True the mutation rate was slower than that obtained by direct irradiation, but it is probable that direct irradiation is able to produce mutagenic substances within the nucleus and thereby by-pass the need for penetration. At all events, the present evidence is that mutagens external to the organism can produce changes in the nucleoproteins of the gene which, if not lethal, are duplicated and passed to the progeny. The presence of the mutagenic chemical in the environment increases the mutation rate; one or more of the mutations may result in a variant which, having a selective advantage in that particular environment may outgrow its non-mutant fellows. In this way the environment can affect the genes even though the mutations produced are wholly at random.

Suggestions that the environment can influence the nature or direction of mutation have, up to now, been stoutly opposed by geneticists. This opposition rests mainly on the evidence, mentioned above, of the stability of the gene in sexually reproducing organisms. Yet mutation must have a biochemical basis and its causes will presumably be found to be chemical in character. What evidence is to be found to support this statement among other organisms? A first example is provided by the phenomenon of enzyme induction. Monod and his colleagues of the Institut Pasteur (41) showed that certain strains of *E. coli* were able to synthesize the enzyme β -galactosidase in response to the presence in the culture medium of certain galactosides. A related phenomenon is the penicillin-induced synthesis of penicillinase by *Bacillus cereus* examined by Pollock (48). The inductor need not, as in the latter example, be identical to the substrate, for certain β -thiogalactosides induce the formation of β -galactosidase, yet are not hydrolysed by the induced enzyme.

The mechanism of enzyme induction discussed by Monod (40) requires examination. When an inductor, for example, methyl- β -D-thiogalactoside, is added in saturating concentrations (ca $5 \times 10^{-4}M$) to a culture of cells with succinate as substrate, the rate of increase of galactosidase in relation to the bacterial mass is constant. If, however, the cells are exposed to lower concentrations of inductor, the rate of enzyme synthesis then slowly increases to a uniform rate as if it has taken time for an effective concentration of inductor to accumulate within the cells. But if these cells are washed to remove the inductor and allowed to regrow, the inductor at low concentrations is able to initiate enzyme synthesis immediately at a constant rate. To explain this "pre-induction" effect, Monod postulated another enzyme system "Y" present in induced cells which facilitates the entry of the inductor. The enzyme "Y" is distinct from the galactosidase and is further necessary to explain the inhibitory effect of glucose. Pre-induced cells produce galactosidase over many generations when grown in the presence of glucose and inductor (at low concentrations); on the other hand, initially non-pre-induced cells over the same number of generations on the same medium do not synthesize the enzyme. The Y enzyme therefore perpetuates itself in the growing population of pre-induced cells and, in Monod's words, "may, under certain conditions, tend to mimic genetic transmission of the acquired 'induced' condition."

This induced alteration in the state of the enzyme protein of the organism may be regarded as a concrete example of the suggestion of Sevag (49) that persistent changes in microorganisms such as drug resistance are the result of the altered state of the enzyme proteins, which may invoke alterations in the self-reproducing nucleoproteins themselves. To this latter group belong the genes and viruses.

In a discussion of the possibility that the environment may influence the hereditary mechanisms, the idea that the latter may involve cytoplasmic factors as distinct from nuclear genes should not remain unmentioned. The postulation of genetic determinants not chromosomal in character seems necessary to explain certain experimental results; Crosby (12) discussed a mechanism whereby (if identities such as the plasmagenes* exist) these bodies enable the inheritance of acquired adaptations. The evidence relative to bacteria is discussed by Hinshelwood (27). A practical example from among insects is due to Waddington (57) who exposed 17-23-hour-old pupae of *Drosophila melanogaster* to temperatures of 40°C. for four hours. About 48 per cent of the flies which subsequently emerged were crossveinless. Breeding was continued from normal and crossveinless stocks and selection continued until, from the twelfth generation onwards, crossveinless flies were produced from pupae not given the heat treatment. In Waddington's words, "During the course of selection, a genetic constitution has therefore been synthesized which, under normal conditions, produced the same effect as was originally found only as a response to the stimulus of an abnormal environment".

But the experimental data of bacteriology are still too incomplete to permit even a tentative summing-up of the role, direct or indirect, of chemical environment on dissociation. Bacterial transformation and transduction are, however, particular cases of dissociation which warrant separate examination for their mechanics are better known.

It is already evident that the experimental test of the influence of environment in heredity may first come from the viruses. The tobacco mosaic virus, first obtained in crystalline form by Stanley (52) in 1935, is a rod-shaped particle of about 300 m μ long and 15 m μ in diameter (Williams and Steere, 58). It consists of a double-walled cylinder of protein constituting about 94 per cent of the weight of the particle and an inner rod-like core of nucleic acid. As in the other plant viruses examined, the nucleic acid component is wholly ribonucleic acid, whereas that of bacteriophages is deoxyribonucleic acid. The nucleic acid content of the plant viruses may range up to about 40 per cent. (see Knight, 34). The virus protein yields no unusual amino acid, and only the L-isomers appear to be present. By gentle chemical treatment, Fraenkel-Conrat and Williams (20) have been able to separate the protein and nucleic acid components of tobacco mosaic virus, each of which alone is almost non-infective. Yet, when mixed in slightly acid solution, the two components recombine to form particles not distinguishable from the original virus and with a similar though weaker pathogenicity to tobacco—yet still two hundred times that of the nucleic acid component. By improving the conditions for recombination, Commoner and his colleagues (11a) raised the infectivity of the reconstituted virus to about one-tenth that of the original virus.

Many strains of the tobacco mosaic virus are known. They have different virulence or infective power, and arise by processes analogous to mutation. Passage of a plant virus through a new host, or the subjecting of the infected host to unusual environmental conditions, may produce such a change or may select a pre-existent mutant. Bawden (7) reported the reversible change of tobacco mosaic virus from the "bean" form to the "tobacco" form by passage through the appropriate host. As his attempts to gain evidence that his inoculum contained two variants multiplying differentially in the two hosts failed, he concluded that the change was host-induced.

*Danielli (13) has recently suggested that this type of hypothetic determinant of cytoplasmic inheritance be termed a homeostat, defined as "an organization of macromolecules, or of processes, which is self-reproducing and which may (and usually does) determine and control the expression of one or more characters in a cell lineage".

Chemically, each variant of tobacco mosaic virus was shown by Black and Knight (9) to have a characteristic composition, the major differences being in the make-up of the protein component. These differences appeared mainly in the proportion of the amino acids present, though one strain possessed histidine and methionine which were absent in other strains. All thirteen variants, including this one, have similar carboxyl terminal end groups, for treatment with the enzyme carboxypeptidase released only threonine (Knight, 34). No differences could be established in the nucleic acids of the various strains of tobacco mosaic virus, though wide differences were found in the nucleic acids of different viruses.

That differences do exist between the nucleic acids of the various strains of tobacco mosaic virus is elegantly shown by Fraenkel-Conrat and Williams (18, 20). They separated, by the methods mentioned above, the protein and the nucleic acid components of an ordinary strain of the virus and a strain (HR) first isolated from Holmes ribgrass. The latter strain yields a protein differing from that of the ordinary strain in amino acid composition and in antigenic specificity. They reconstituted the virus from the nucleic acid of the HR strain and from the protein of the ordinary strain. This "hybrid" virus when treated with the antiserum of the protein of the ordinary strain lost its infectivity, but not when treated with the antiserum of the HR protein. Hence the hybrid virus protein is that of the ordinary strain. Plants infected with the hybrid virus developed the symptoms of the HR strain and when the progeny of the "hybrid" virus was recovered from the diseased plant, the virus proved to be wholly HR strain, for its protein, like HR protein and unlike the protein of the ordinary strain, contained methionine and histidine and much tyrosine. It is therefore evident that the symptoms produced by the virus are determined by its nucleic acid component, and that there must be differences in the nucleic acids of the different strains. Moreover, the experiment demonstrates that the nucleic acid component controls the synthesis of the protein.

In a semi-popular account of this work, Fraenkel-Conrat (19) wrote that there seem to be slight quantitative differences between the protein of the original HR virus and that of the hybrid virus after passage through the host plant. If these differences are real, the significance of the work becomes even more striking, for it would indicate that the protein of the ordinary strain used for the reconstitution of the virus has had a genetic effect, bringing about a modification of the protein in the progeny of the "hybrid" virus. This would be the first demonstration of a mutation achieved through the mechanism postulated by Sevag, whereby the altered state of the enzyme protein has invoked alteration in the self-producing nucleoproteins.

This interaction of protein and nucleic acid places the phenomenon in a somewhat different category from that displayed under bacterial transduction and transformation in which, as will be shown, the nucleic acids themselves are the agents involved in the hereditary changes induced by the chemicals of the environment.

Bacterial Transformation:

Pneumococci may be broadly divided into two classes: those with polysaccharide capsules (S-type) and those lacking a polysaccharide capsule (R-type). The R-types are generally avirulent for mice, but in 1928 Griffith (25) found that if heat-killed S-type bacteria were added to an inoculum of living R-type cells, infection resulted. Viable S-type cells of the same type as the dead cells added to the inoculum were recovered from the blood of the

infected mice. Although Griffith was unable to duplicate this process *in vitro*, he recognized that material from the killed S-cells had changed the R-type cells to S-type cells.

Conditions necessary for achieving this transformation *in vitro* were quickly found, and Avery and his co-workers succeeded in extracting and purifying the compounds responsible for this drastic alteration of the properties of the pneumococcus. With McCarty (6) they showed that this compound was a highly polymeric and viscous form of sodium deoxyribonucleate of molecular weight of the order of five hundred thousand and active at a concentration of 1 part in 6×10^8 . Hotchkiss (28), by growing penicillin-sensitive pneumococci in the presence of deoxyribonucleate from penicillin-resistant strains, produced penicillin-resistant organisms in a proportion 10,000 times greater than that formed by spontaneous mutation. Similarly, streptomycin-resistant strains of *Haemophilus influenzae* were produced by Alexander and Leidy (3) by the use of deoxyribonucleic acid extracted from streptomycin-resistant *Haemophilus*. Transformation reactions therefore have a wide biological significance—a point stressed by Austrian (5) in his review of the experimental work on this subject. To the examples discussed by Austrian may be added “the tumor-inducing principle” which was postulated by Braun and his colleagues to explain the results of their studies on crown gall (see p. 73).

In all cases the new characters acquired by treatment with the transforming agent were heritable. The transforming agent, of which the physical properties have been reviewed by Zamenhoff (63), appears to be polymeric deoxyribonucleic acid, for its activity is destroyed by deoxyribonuclease. It would seem to be derived from genetic material and to have survived the process of purification, retaining the ability to reach and become incorporated with the genetic mechanism of the bacterium. A remarkable feature is not so much that the chemical responsible for the hereditary change is a large molecule likely to be labile if deprived of its protein protection, but that it can penetrate deeply enough to be incorporated into the cell nucleus. The chemical reactivity of the transforming agent has been investigated by Zamenhoff and his colleagues (64). The agent is resistant to inactivation by protein denaturants but is readily affected by mutagens, undergoing a diversity of reactions—which suggests that different mutagens have different reactions. Zamenhoff's results indicate that the first reaction of the mutagen on the transforming agent is to convert it to an unstable form.

Bacterial Transduction:

A further mechanism by which genetic traits can be transferred from one organism to another was described by Zinder and Lederberg (65). They found that when *Salmonella typhimurium* is grown in the presence of particular temperate phages, it produces a filtrable agent capable of inducing hereditary changes in other strains of the bacillus, much after the manner of transformation. Unlike the transformation agent, however, this filtrable agent is not affected by deoxyribonuclease, nor is it readily extractable from the cell. Its particles are about 100 m μ in diameter and appear to be carried on the phage. This phenomenon, whereby heritable characters are transferred from donor bacteria to recipient bacteria, was called “transduction” by Zinder and Lederberg (65). They then, and later with Stocker (53), suggested that transduction results from the transfer of genetic material by particles of “filterable agent”. The material transferred may well be deoxyribonucleic acid, but here it is protected by the protein of the phage particles and it is not free in the medium as in the case of transformation. Moreover the phage here provides the means of entry into the transduced cell.

The fast-accumulating experimental evidence of bacterial and virus dissociation seems to reveal that the gene is but a chemical, a complex of nucleic acid and protein, by no means immune from reaction with the components of its environment. The manipulation of the chemistry of the nucleic acids in the living organism, with a maintenance of the *in vivo* conditions, will clearly be of extreme difficulty. But the prospect of the purposeful induction of non-lethal mutations provides one of the most thrilling vistas of ecological chemistry.

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AUTHOR INDEX

- | | | | |
|--------------------------|---|------------------------|------------------------|
| Abraham, E. P. | 86 | Broadfoot, W. C. | 53, 66 |
| Ackerman, L. | 30 | Brown, J. J. | 58, 64 |
| Acree, F. | 69, 70, 71 | Brown, R. | 15, 18 |
| Alcorn, S. M. | 63 | Brown W. | 43, 48, 50 |
| Alderton, G. | 85 | Bruce, W. F. | 55, 64, 65 |
| Alexander, H. E. | 84, 85, 87 | Buchanan, J. B. | 64, 65 |
| Alexander, P. | 69, 70 | Burnet, F. M. | 78, 85 |
| Amoureux, G. | 73, 75 | Calam, C. T. | 18, 19 |
| Anderson, J. A. | 33, 36 | Cameron, F. K. | 8, 19 |
| Andreae, W. A. | 17, 18 | Carter, W. | 42 |
| Angus, T. A. | 68, 69, 71 | Cation, D. | 65 |
| Arens, K. | 36 | Cavill, G. W. K. | 67, 71 |
| Ark, P. A. | 63 | Chabrolin, C. | 15, 19 |
| Ashton, G. C. | 57, 63 | Chadwick, L. E. | 23, 24, 30, 70, 71 |
| Aso, K. | 52 | Chen, H. K. | 73, 75 |
| Atkinson, R. G. | 61, 65 | Chinn, S. H. F. | 62, 64 |
| Auclair, J. L. | 27, 30 | Chittenden, F. H. | 69, 71 |
| Auerbach, C. | 80, 85 | Clark, R. V. | 51 |
| Austrian, R. | 84, 85 | Clauson-Kaas, N. | 47, 50 |
| Avery, O. T. | 84, 85 | Clements, F. E. | 6, 11, 19 |
| Baker, F. C. | 29, 30 | Cohen-Bazire, E. | 86 |
| Barcinsky, R. | 15, 18 | Cohn, M. | 86 |
| Barnes, R. A. | 36 | Collins, R. P. | 48, 51 |
| Barnes, T. W. | 9, 11, 20 | Commoner, B. | 82, 85 |
| Barnhardt, C. S. | 70, 71 | Conway, H. F. | 52 |
| Barton, D. H. R. | 69, 70 | Cook, A. H. | 48, 50 |
| Baunacke, W. | 15, 18 | Cormack, M. W. | 14, 19 |
| Bawden, F. C. | 59, 63, 82, 85 | Cornfeld, E. C. | 59 |
| Bazzigher, G. | 44, 50 | Cox, S. F. | 50 |
| Beard, R. L. | 68, 71 | Craig, L. C. | 77, 86 |
| Beck, E. G. | 41, 42 | Craighead, F. C. | 22, 30 |
| Becker, Y. | 18 | Crombie, A. C. | 23, 30, 32 |
| Bedford, Duke of | 20 | Crosby, J. L. | 82, 85 |
| Benedict, H. M. | 13, 18 | Cross, B. E. | 45, 46, 50 |
| Bennett, E. L. | 12, 18 | Crowdy, S. H. | 62, 64 |
| Bentley, J. A. | 17, 18 | Curtis, P. J. | 45, 50, 56, 57, 58, 63 |
| Bergold, G. H. | 79, 85 | Cushing, J. E. | 71 |
| Berridge, N. J. | 86 | Danielli, J. F. | 82, 85 |
| Berthelot, A. | 73, 75 | Darpoux, H. | 44, 50, 51 |
| Bickle, A. S. | 17, 18 | Darrah, J. H. | 32 |
| Bishop, D. | 16, 18 | Daubeny, C. G. B. | 8, 19 |
| Black, F. L. | 83, 85 | Davenport, O. | 69, 71 |
| Bliss, D. E. | 61, 63 | Davidson, J. | 27, 28, 30 |
| Blondeau, R. | 13, 21 | Davis, E. | 12, 19 |
| Bode, N. R. | 13, 18 | Dawson, R. F. | 27, 30 |
| Bonner, J. | 12, 13, 18, 19 | de Candolle, A. | 7, 9, 14 |
| Bonner, J. T. | 70, 71 | De Hahn, J. T. | 45, 51 |
| Borthwick, H. A. | 32 | Dekker, J. | 62, 64 |
| Boussingault, J. B. | 7 | De Kruif, P. H. | 79, 85 |
| Boysen Jensen, P. | 41, 42 | Delbrück, M. | 80 |
| Braun, A. C. | 73, 74, 75, 76, 84 | Deleuil, G. | 12, 19 |
| Brian, P. W. | 43, 44, 46, 47, 48, 50, 54,
56, 57, 58, 59, 62, 63, 64 | De Ropp, R. S. | 73, 75 |
| Brierley, W. B. | 36, 79, 85 | Dethier, V. G. | 22, 23, 24, 30, 70, 71 |
| Broadbent, L. | 22, 30 | De Vries, H. | 80, 85 |
| | | Dickson, J. G. | 14, 20 |

AUTHOR INDEX

- Dickson, R. C. 28, 30
 Dienes, L. 80, 85
 Dimond, A. E. 43, 47, 48, 49, 51, 52
 Doolittle, S. P. 34, 36
 Dufrénoy, J. 33, 36, 50, 51
 Duley, F. L. 13, 20
 Durrell, L. W. 36
 Dutcher, J. D. 55, 64
 Eberhardt, F. 17, 19
 Elson, G. W. 50
 Elvidge, J. A. 55, 64
 Emerson, O. H. 55, 66
 Enderlein, G. 79, 85
 Erdtman, H. 36
 Evans, A. C. 27, 30
 Evelyn, J. 6, 11
 Evanari, M. 16, 17, 19
 Faivre-Amiot, A. 44, 50, 51
 Falck, R. 54, 64
 Falconer, D. S. 24, 30
 Farmer, T. H. 50
 Fawcett, H. S. 53, 64
 Felber, I. M. 64
 Fennell, D. I. 52
 Fitz-James, P. 69, 71
 Flanders, S. F. 25, 30
 Florey, H. W. 77, 86
 Folsom, J. W. 24, 30
 Fontaine, T. D. 34, 36
 Ford, D. L. 67, 71
 Ford, J. H. 58, 59, 64, 65
 Forsman, B. 38, 39
 Forsyth, F. R. 43, 51
 Fraenkel, G. 26, 27, 30, 31
 Fraenkel-Conrat, H. 82, 83, 85
 Fredericq, P. 77, 86
 Freeman, G. G. 59, 63, 64
 Freeman, V. J. 78, 86
 Fries, N. 38, 39
 Frisch, K. von 24, 31
 Fujiki, T. 79, 87
 Fukunaga, K. 52
 Funke, G. 13, 19
 Fusco, F. 67, 71
 Galston, A. W. 13, 18
 Gardner, Joan F. 77, 86
 Gauhe, A. 36
 Gäumann, E. 33, 34, 36, 43,
 45, 47, 48, 50, 51
 Gerber, N. N. 36
 Gill, J. E. 36, 59, 64
 Gilmore, A. E. 14, 20
 Glistler, G. A. 64
 Goodwin, R. H. 19
 Gothoskar, S. S. 48, 51
 Gottlieb, D. 60, 64, 66
 Gough, H. C. 69, 71
 Gratia, A. 77, 85
 Gray, R. 12, 13, 19
 Greenwood, A. D. 18
 Grevillius, A. Y. 24, 31
 Gries, G. A. 12, 19
 Griffith, F. 83, 86
 Gripenberg, J. 36
 Grossbard, E. 60, 61, 64
 Grove, J. F. 44, 50, 51, 56, 57, 58, 63, 64
 Guldensteeden-Egeling, C. 69, 71
 Gutmann, A. 78, 86
 Guyot, L. 12, 18, 19
 Haagen-Smit, A. J. 13, 19
 Haas, F. 81, 87
 HacsKaylo, E. 50, 51
 Hahn, E. 87
 Haller, H. L. 69, 71
 Hamner, C. L. 64
 Hannay, C. L. 68, 69, 71
 Hartland, B. L. 9, 19
 Hatfield, W. C. 36
 Hayashi, T. 45, 47, 52
 Heatley, N. G. 77, 86
 Hellriegel, H. 7, 19
 Hemberg, T. 17, 19
 Hemming, H. G. 47, 50, 56, 57, 58,
 59, 62, 63, 64
 Hendricks, S. B. 32
 Hessayon, D. G. 60, 64
 Hieke, K. 27, 31
 Hietala, P. K. 34, 37
 Hiltner, L. 38, 39
 Hijner, J. A. 16, 19
 Hildebrand, A. A. 60, 66
 Hill, R. 32
 Hinshelwood, C. N. 82, 86
 Hoogenheide, J. C. 65
 Hopkins, A. D. 22, 31
 Hopkins, B. A. 28, 32
 Horiuchi, M. 52
 Horne, W. 13, 21
 Hotchkiss, R. D. 84, 86
 Howland, R. B. 69, 71
 Howland, S. R. 56, 66
 Hunter, A. 11
 Husain, M. A. 25, 31
 Igarashi, H. 52
 Irving, G. W. 34, 36
 Jackson, R. W. 52
 Jacob, F. 77, 78, 86
 James, R. G. 59, 65
 James, W. O. 27, 31
 Jefferys, E. G. 60, 62, 64
 Jenner, C. E. 32
 Jerrel, E. A. 68, 71
 Johansen, F. D. 14, 19
 Johnson, A. W. 15, 18
 Johnson, B. 31
 Johnson, J. 74, 75
 Johnson, J. R. 54, 55, 56, 64, 65
 Jones, F. G. W. 16, 19, 23, 32
 Juhren, G. 17, 21
 Juhren, M. C. 17, 21
 Julien, J. B. 51
 Kaji, J. 45, 52

AUTHOR INDEX

- | | | | |
|-----------------------------|----------------|-------------------------------|----------------|
| Kambe, K. | 45, 52 | Marrian, D. H. | 20 |
| Katznelson, H. | 39 | Martin, J. P. | 41, 42 |
| Kenten, J. | 28, 31 | Massenot, M. | 18 |
| King, H. E. | 25, 32 | Massey, A. B. | 12, 20 |
| Klein, D. T. | 73, 75 | McCalla, T. M. | 13, 20 |
| Klein, R. M. | 73, 75, 76 | McCarty, M. | 84, 85 |
| Kleineberger-Nobel, E. | 80, 86 | McColloch, J. W. | 23, 31 |
| Knight, C. A. | 82, 83, 85, 86 | McCrone, W. C. | 55, 65 |
| Kobel, H. | 45, 51 | McElroy, W. D. | 69, 71 |
| Koch, L. | 15, 20 | McGowan, J. C. | 56, 57, 62, 63 |
| Koch, L. W. | 14, 20, 59, 65 | McIndoo, N. E. | 24, 31 |
| Koeckemann, A. | 16, 20 | McKie, M. | 78, 85 |
| Kojima, Y. | 78, 86 | McKinney, K. B. | 31 |
| Kornfeld, E. C. | 59, 65 | Mebane, A. D. | 65 |
| Kuenen, D. J. | 29, 31 | Melin, E. | 62, 65 |
| Kuhn, R. | 25, 26, 31, 34 | Menzel, A. E. O. | 65 |
| Kurokawa, M. | 78, 86 | Miescher, G. | 48, 51 |
| Kurosawa, E. | 45, 51 | Millard, W. A. | 53, 65 |
| Küster, E. | 41, 42 | Mitsuhashi, S. | 78, 86 |
| Lackmann, J. | 7, 20 | Moewus, L. | 16, 20 |
| Lal, K. B. | 25, 31 | Moewus, T. | 16, 20 |
| Langford, C. T. | 52 | Molisch, H. | 11, 20 |
| Lansdown, A. R. | 18 | Monod, J. | 81, 86 |
| Laskaris, T. | 73, 75 | Montégut, J. | 18 |
| Lathrop, F. H. | 27, 31 | Montieth, L. G. | 23, 31 |
| Leach, B. E. | 58, 64, 65 | Morrison, R. I. | 59, 64 |
| Lebeau, J. B. | 14, 20 | Mulholland, T. P. C. | 46, 50, 51, 64 |
| Lederberg, J. | 84, 87 | Mull, R. P. | 65 |
| Lees, A. D. | 29, 31 | Muller, C. H. | 13, 20 |
| Leidy, G. | 84, 85, 87 | Muller, H. J. | 80, 86 |
| Levan, A. | 80, 86 | Murwin, H. F. | 74 |
| Levison, I. | 50, 51 | Naef-Roth, St. | 45, 48, 51 |
| Lewis, I. F. | 41, 42 | Nager, W. | 48, 51 |
| Liebig, J. von | 8, 20 | Neigisch, W. A. | 69, 71 |
| Liener, I. E. | 26, 31 | Neilson-Jones, W. | 61, 65 |
| Lindley, J. | 14, 20 | Newbold, G. T. | 58, 64 |
| Link, G. K. K. | 73, 76 | Newcomb, E. H. | 42 |
| Lipke, H. | 26, 31 | Newcombe, H. B. | 80, 86 |
| Lipp, J. W. | 23, 31 | Newton, G. G. F. | 86 |
| Lippincott, J. A. | 85 | Newton, R. | 33, 36 |
| Lockhead, A. G. | 38, 39 | Nicol, H. | 73, 76 |
| Locksley, H. D. | 67, 71 | Nutman, P. S. | 38, 39 |
| Loconti, J. D. | 69, 71 | Olive, E. W. | 70, 71 |
| Loehwing, W. F. | 9, 20 | Oroshnik, W. | 62, 65 |
| Long, A. G. | 18 | Ostvald, H. | 10, 20 |
| Löw, I. | 25, 31, 34 | Owen, J. H. | 36, 37 |
| Lowe, D. | 62, 64 | Oxford, A. E. | 56, 65 |
| Luckwill, L. C. | 40, 42 | Painter, R. H. | 22 |
| Ludwig, R. A. | 43, 44, 48, 51 | Paladini, A. | 77, 86 |
| Luria, S. E. | 80, 86 | Pallansch, M. J. | 31 |
| Lwoff, A. | 78, 86 | Palmer, J. G. | 50 |
| Lyon, T. L. | 38, 39 | Pappenheimer, A. M., Jr. | 78, 79, 86 |
| Ma, R. | 36 | Parke, T. V. | 59, 65 |
| MacDougall, D. T. | 49, 51 | Parker, M. W. | 29, 32 |
| Machacek, J. E. | 53, 65 | Parkinson, D. | 39 |
| MacLeod, C. M. | 85 | Parnell, F. R. | 25, 32 |
| MacMillan, J. | 50, 57, 64, 65 | Parr, T. | 42 |
| Malpighi, M. | 7 | Patrick, Z. A. | 14, 20 |
| Maltais, J. B. | 27, 31 | Payne, T. M. B. | 39 |
| Mann, H. H. | 9, 11, 20 | Peterson, D. | 65 |
| Marcovitch, S. | 28, 31 | Pickering, S. U. | 10, 11, 20 |

AUTHOR INDEX

- | | | | |
|------------------------------|----------------|----------------------------|----------------|
| Pierson, C. F. | 48, 51 | Skinner, J. J. | 8, 21 |
| Pilet, P. E. | 49, 51 | Skolko, A. J. | 66 |
| Plattner, P. A. | 48, 50, 51 | Slankis, V. | 50, 51 |
| Pollock, M. R. | 81, 86 | Sloan, H. | 11 |
| Poole, J. B. | 36 | Smith, K. M. | 79, 87 |
| Póts, S. F. | 69, 71 | Sohns, V. E. | 52 |
| Pound, G. S. | 44, 51 | Sowder, A. M. | 35, 37 |
| Pramer, D. | 62, 64, 65 | Spring, F. S. | 55, 64 |
| Proebsting, E. L. | 14, 20 | Stahl, W. H. | 69, 71 |
| Radley, M. | 50 | Stahmann, M. A. | 37, 51 |
| Raistrick, H. | 18, 56, 58, 65 | Stanley, W. M. | 82, 87 |
| Raper, K. B. | 52 | Steere, R. L. | 82, 87 |
| Raudnitz, H. | 56, 66 | Steeves, T. A. | 49, 51 |
| Rayner, M. C. | 61, 65 | Steinhaus, E. A. | 68, 71 |
| Reed, H. S. | 21 | Stephens, J. M. | 71 |
| Reid, R. D. | 87 | Stevenson, I. L. | 61, 66 |
| Reinhardt, H. J. | 32 | Stewart, W. S. | 17, 21 |
| Rennerfelt, E. | 36, 37 | Stiven, G. | 13, 21 |
| Reynolds, O. E. | 87 | Stocker, B. A. D. | 84, 87 |
| Rhodes, A. | 57, 63 | Stodola, E. H. | 46, 52 |
| Richardson, H. H. | 32 | Stokes, A. | 62, 66 |
| Richardson, L. T. | 61, 65 | Stone, W. S. | 81, 87 |
| Richman, E. E. | 85 | Strehler, B. L. | 69, 71 |
| Ridé, M. | 44, 51 | Stringer, A. | 32 |
| Riker, A. J. | 73, 76 | Stubbs, J. | 64 |
| Ripley, P. O. | 9, 11, 20 | Sumuki, Y. | 52 |
| Rivnay, E. | 27, 32 | Sunderland, N. | 18 |
| Roberts, E. A. | 38, 39 | Sussman, M. | 70, 72 |
| Robinson, D. B. | 51 | Sutton, M. D. | 66 |
| Robinson, E. | 15, 18 | Swarbrick, T. | 40, 42 |
| Robson, J. M. | 80, 85 | Taber, W. A. | 65 |
| Ross, D. J. | 58, 65 | Tamari, K. | 45, 52 |
| Roth, L. M. | 69, 71 | Tamura, T. | 52 |
| Rouatt, J. W. | 39, 61, 65 | Taves, C. | 19 |
| Roux, E. R. | 13, 21 | Taylor, C. B. | 53, 65 |
| Roux, L. | 44, 50, 51 | Tetjurev, W. A. | 16, 21 |
| Rovina, A. D. | 38, 39 | Thexton, R. H. | 39 |
| Ruhnke, G. N. | 14, 21 | Thimann, K. V. | 73, 76 |
| Russell, E. J. | 8, 9, 11, 21 | Thomas, J. E. | 73, 76 |
| Russell, P. B. | 20 | Thornton, H. G. | 73, 76 |
| Russell, R. C. | 62, 64 | Thorold Rogers, M. A. | 64 |
| Ruston, D. F. | 25, 32 | Thorpe, W. H. | 23, 24, 32 |
| Salt, G. | 25, 32 | Thorsteinson, A. J. | 24, 32 |
| Sanders, E. | 28, 30 | Timonin, M. I. | 14, 21 |
| Sanford, G. B. | 53, 65, 66 | Todd, A. R. | 15, 18, 19, 20 |
| Saunders, A. R. | 15, 21 | Topley, W. W. C. | 75, 76 |
| Schader, E. | 16, 20 | Toumanoff, C. | 72 |
| Schaffner, G. | 74, 76 | Townley, R. W. | 65 |
| Schearer, G. B. | 85 | Trave, R. | 67, 71 |
| Scheffer, R. P. | 48, 51 | Trischmann, H. | 34, 36 |
| Schlectendahl, D. F. C. | 41, 42 | Tyler, G. J. | 18 |
| Schneiderhan, F. J. | 12, 21 | Unwin, C. H. | 50 |
| Scholz, C. R. | 65 | Upshall, W. H. | 14, 21 |
| Schreiner, O. | 8, 21 | Vago, C. | 72, 79, 87 |
| Sequeira, L. | 49, 51 | Van Heyningen, W. E. | 77, 87 |
| Sevag, M. G. | 81, 83, 87 | Van Overbeek, J. | 13, 21 |
| Shaffer, B. M. | 70, 71 | Vasudeva, R. S. | 53, 66 |
| Shirozu, Y. | 79, 87 | Vercellone, A. | 67, 71 |
| Siminoff, P. | 60, 64, 66 | Vershaffelt, E. | 24, 32 |
| Siminovitch, L. | 77, 86 | Vining, L. C. | 65 |
| Simonart, P. | 56, 65 | Virtanen, A. I. | 34, 37 |

AUTHOR INDEX

- | | | | |
|----------------------------|------------------------|------------------------|--------------------|
| Vischer, E. B. | 56, 66 | Williams, C. M. | 79, 86 |
| Vozniakovskaia, Y. M. | 38 | Williams, R. C. | 82, 83, 85, 87 |
| Waddington, C. H. | 82, 87 | Williams, T. L. | 64 |
| Waggoner, P. E. | 43, 47, 48, 49, 51, 52 | Williamson, C. E. | 49, 52 |
| Waksman, S. A. | 54, 66 | Wilson, F. | 28, 32 |
| Walker, J. C. | 33, 36, 37, 48, 51, 52 | Wilson, G. S. | 75, 76 |
| Wallace, R. H. | 38, 39 | Wilson, P. K. | 38, 39 |
| Wallen, V. R. | 62, 66 | Winslow, R. D. | 15, 21 |
| Walsh, B. D. | 22, 32 | Winstead, N. N. | 48, 52 |
| Walton, L. | 41, 42 | Wintersteiner, O. | 65 |
| Ward, C. | 46, 51 | Wolf, F. T. | 49, 52 |
| Waring, W. S. | 19, 20 | Wollman, E. | 86 |
| Way, A. M. | 64 | Wollman, E. L. | 77, 78, 86 |
| Way, M. J. | 28, 32 | Woolley, D. W. | 47, 52, 74, 76 |
| Weinberger, H. I. | 80, 85 | Wootten, D. M. | 71 |
| Weindling, R. | 53, 54, 66 | Wright, J. M. | 50, 60, 61, 64, 66 |
| Wellman, F. L. | 49, 52 | Wu, Jia-Hsi | 85 |
| Went, F. W. | 12, 13, 16, 17, 19, 21 | Wyckoff, R. W. G. | 79, 87 |
| West, P. M. | 38, 39, 60, 66 | Wyss, O. | 81, 87 |
| Whetzel, H. H. | 59, 66 | Yabuta, T. | 45, 46, 47, 52 |
| Whiffen, A. J. | 58, 65 | Yamafugi, K. | 79, 87 |
| White, P. R. | 73, 75 | Yoshihara, I. | 79, 87 |
| Whitney, M. | 8, 21 | Zamenhof, S. | 84, 87 |
| Wilfarth, H. | 7, 19 | Zinder, N. D. | 84, 87 |

SUBJECT INDEX

- | | | | |
|--|------------|--|-----------|
| 3-Acetyl-6-methoxybenzaldehyde | 12, 13 | Benzoic acid, as phytotoxin | 14 |
| <i>Achroia grisella</i> | 23 | Benzoxazolinone, as fungicide | 34 |
| Acrasins | 70 | <i>Beta patellaris</i> | 16 |
| "Acti-dione" | 58 | <i>Beta webbiana</i> | 16 |
| <i>Actinomyces scabies</i> | 53 | "Big Bud" of currants | 40 |
| <i>Acyrtosiphon pisum</i> | 27, 28 | Biotin, as root exudate | 38 |
| Adsorption, removal of toxins by | 8 | Blastocholines | 16 |
| <i>Agriotes</i> spp., food finding by | 24 | <i>Boletus bovinus</i> | 62 |
| <i>Agrobacterium radiobacter</i> | 73 | <i>Boletus luteus</i> | 50 |
| <i>Agrobacterium rubi</i> | 73 | <i>Boletus variegatus</i> | 50 |
| <i>Agropyron repens</i> | 10 | <i>Bombyx mori</i> | 68, 79 |
| <i>Agrostis gigantea</i> | 9 | <i>Brevicoryne brassicae</i> , photoperiod- | |
| Aldehydes, as phytotoxins | 8 | ism in | 27 |
| Alkaloids, biosynthesis of | 27 | Bromegrass, phytotoxins of | 13 |
| Allelopathy | 11 | <i>Bromus inermis</i> | 13 |
| Allogibberic acid | 46 | Browntail moth, host selection by | 24 |
| <i>Alternaria solani</i> | 44 | Butoxypolypropylene glycol, as | |
| Alternaric acid | 44 | insect repellent | 25 |
| Alternarin | 44 | <i>t</i> -Butyl peroxide, as mutagen | 78 |
| <i>Amaranthus hybridus</i> | 17 | 3-Butylpyridine-6-carboxylic acid | 45 |
| Amino acids, excretion from plant | | Cabbage butterflies, chemotropic | |
| roots | 38 | responses of | 24 |
| Amino acids, factors in insect resist- | | <i>Carpocapsa pomonella</i> | 68 |
| ance | 27 | <i>Castanea dentata</i> | 44 |
| Amygdalin, as phytotoxin | 14 | <i>Castanea sativa</i> | 44 |
| <i>Andropogon pubescens</i> | 12 | Catechol, as fungicide | 33 |
| <i>Anthonomus grandis</i> | 24 | Chalcones, as factors in resistance | 26 |
| Antibiosis | 5 | <i>Chenopodium album</i> , inhibition by | |
| Antibiotics | 54, 60 | barley | 17 |
| <i>Anuraphis roseus</i> | 28, 40 | Chickweed, effect on crop plants | 9 |
| Aphids, photoperiodism in | 28 | Chloramphenicol | 60, 62 |
| <i>Aphis craccivora</i> | 25 | <i>Chlorida obsoleta</i> , host selection by | 23 |
| <i>Aphis chloris</i> | 28 | Chloromycetin | 60 |
| <i>Aphis rumicis</i> | 28 | <i>Cibotium Barometz</i> | 11 |
| <i>Aphis sorbi</i> | 28 | Cinnamic acids, as phytotoxins | 13 |
| <i>Armillaria mellea</i> | 61 | <i>Cladosporium fulvum</i> , in antibiosis | 53 |
| <i>Artemisia absinthium</i> | 13 | Coffee leaf disease | 49 |
| <i>Artemisia vulgaris</i> | 13 | <i>Colias philodice eurytheme</i> , bio- | |
| Asparagine, as insect attractant | 24 | logical control of | 68 |
| <i>Aspergillus helvola</i> | 55 | Colicines | 77 |
| <i>Aspergillus fumigatus</i> | 55 | <i>Colletotrichum circinans</i> | 33 |
| <i>Asterolecanium variolosum</i> | 42 | Colorado potato beetle, host re- | |
| <i>Bacillus cereus</i> | 68, 81 | sistance to | 25, 34 |
| <i>Bacillus sotto</i> | 68 | Competition, among higher plants | |
| <i>Bacillus thuringiensis</i> | 68 | | 6 et seq. |
| Bacterial dissociation | 79 et seq. | Corn earworm, host selection by | 23 |
| Bacterial transduction | 84 | <i>Coronopus squamatus</i> , use against | |
| Bacterial transformation | 83 | eelworms | 16 |
| Bacteriocinogenicity | 78 | <i>Corynebacterium diphtheriae</i> | 68, 78 |
| Bacteriocins | 77 | Couch grasses, phytotoxicity of | 10 |
| Bacteriophages | 79 | Coumarins, as phytotoxins | 13 |
| <i>Bacterium tumefaciens</i> | 73 | Crop rotation | 7 |
| Barberry, effect on wheat | 7 | Crown gall | 73 |
| Barley, effect of grasses on | 9 | "Curling Factor" | 56 |
| Bees, chemotropic responses of | 24 | Cyclogeny | 79 |

SUBJECT INDEX

- L- forms of bacteria 80
 Linseed, seed germination stimulant
 from 15
Lithospermum fruticosum 12
Lycomarasmin 47
Lycopersicin 34
Lycopersicum pimpinellifolium ... 34
Lymantria dispar 69
 Lysogeny 78
Matricaria inodora 9
 Meadow nematodes 14
Metatechanychus ulmi, photoperiod-
 ism in 29
 Methyl bis (β -chloroethyl) amine,
 as mutagen 78
 2-Methyl-1,4-dihydroxybenzene,
 from insects 69
Mikiola fagi, gall formation by 41
 Monoculture, defects of 9
 Mouse-ear, phytotoxins from 12
Musca domestica 70
 Mustard oils, as insect attractants .. 24
 Mutation 80
Mycorrhiza 49, 61
Myzus persicae 22, 25
 Naphthaleneacetic acid 40
 Nematodes, stimulation of 15
Nemeritis canescens, host selection
 by 23
Nicotiana tabacum 27
 Nisin 77
 Nitrogen mustards, as mutagens ... 78
 Olfactory responses, in host selection 24
Omphalia flava 49
 Onion, resistance to *Colletotri-*
 chum 33
Ophiobolus graminis 53
 Oriental fruit moth, photoperiod-
 ism in 28
Orobanche cumana 15
Orobanche minor 15
Orobanche speciosa 15
 Osage orange, resistance to fungi .. 36
 Oxytetracycline 62
Parthenium argentatum 13
 Peach, replant problem 7, 14
 Peas, aphid resistance in 27
 Pectolytic enzymes, as phytotoxins. 48
Penicillium cyclopium 58
Penicillium digitalum 59
Penicillium expansum 53
Penicillium gladioli 57, 58
Penicillium griseofulvin 56, 57
Penicillium janczewskii 56, 57
Penicillium jensenii 55
Penicillium obscurum 55
Penicillium patulum 60
Penicillium terlikewskii 55
Phaseolus vulgaris 25
 Phenols, as fungicides 33
Phormia regina 24
 Photoperiodism 28
 o-Phthalaldehyde 58
Phytoptus ribis 40
 Phytotoxins, from higher plants
 11 et seq.
 Phytotoxins, role in plant
 pathology 43 et seq.
Pieris brassicae 24
Pieris rapae 24
 Pinosylvin, as fungicide 34
 Plant excretions, as factor in crop
 rotation 7
 Plant excretions, as source of phyto-
 toxins 8
 Plasmagones 82
Popillia japonica, host selection by 23
 Potato scab, control by green
 manuring 53
 Prophages 78
 Prosoplasmas 41
 Protocatechuic acid, as fungicide .. 33
Pseudomonas angulata 75
Pseudomonas tabaci, toxins from .. 74
Pseudomonas viscosa 62
Puccinia graminis, phytotoxins from 43
 Pyocine 77
Pyrausta nubilalis 68
 Red clover, plowing in of 61
Rhizobium spp. 7, 73
 Rhizosphere 38
Rhyzopertha dominica 23
 Rimocidin 62
 Root excretions, historical 8
 Root excretions, effect on higher
 plants 8 et seq.
 Root excretions, effects on soil
 organisms 38 et seq.
 Rye, resistance to *Fusarium* 34
 Salicylaldehyde, as phytotoxin ... 8
Salmonella spp. 77, 84
Sclerotinia fructicola 53
Sclerotinia fructigena 53
 Scopoletin, as phytotoxin 17
 Scythian lamb 6, 11
 Seed treatment, use of antibiotics for 62
Shigella dysenteriae 77
 Sinalbin, as insect attractant 24
 Sinigrin, as insect attractant 24
 Snow mold 14
 Solanidine 26, 35
 Solanines 26
Solanum andigenum 16
Solanum chacoense 25
Solanum demissum 25, 26
Solanum dulcamara 26
Solanum nigrum 26
Solanum polyadenium 25
Solanum tuberosum 26
Solidago virgaurea 12

SUBJECT INDEX

- | | | | |
|---|--------|---|--------------------|
| Cycloheximide | 58 | <i>Fusarium culmorum</i> | 14 |
| Cyclopolic acid | 58 | <i>Fusarium lini</i> | 14 |
| Cytoplasmic inheritance | 82 | <i>Fusarium lycopersici</i> | 34, 47, 48, 49 |
| 2,4-D | 13, 49 | <i>Fusarium moniliforme</i> | 45 |
| Day length, effects on organisms .. | 29 | <i>Fusarium nivale</i> | 34 |
| Dechlorogriseofulvin | 87 | Gall formation by insects | 41 |
| Demissidine | 35 | <i>Gibberella fujikuroi</i> | 45 |
| Demissine | 26 | Gibberellic acid | 45 |
| Deoxyribonucleic acids | 78, 84 | Gibberellins | 45 |
| Diamond back moth, host selection
by | 24 | Gladiolic acid | 57 |
| Diapause in insects, hormonal con-
trol of | 29 | <i>Gliocladium fimbriatum</i> | 54 |
| Diaporthin | 44 | Glitoxin | 54 et seq. 60 |
| <i>Diataraxia oleracea</i> | 28 | Glucocheirolin, as insect attractant | 24 |
| <i>Dibotryon morbosum</i> | 59 | <i>Gnorimoschema gallaesolidaginis</i> ,
gall formation by | 41 |
| <i>N,N</i> -Diethyl toluamide, as insect
repellent | 25 | Gramicidin | 77 |
| Dihydrogladiolic acid | 58 | <i>Grapholitha molesta</i> , photoperiod-
ism in | 28 |
| Dihydroxystearic acid, as phyto-
toxin | 8 | Grasses, interspecific effects | 9 |
| 3,5-Dihydroxy- <i>trans</i> stilbene, as fun-
gicide | 34 | Griseofulvin | 56 |
| Dimethylfluorene | 46 | Guayule, phytotoxins from | 13 |
| Dimethyl phthalate, as insect re-
pellent | 25 | Gum, as defense against pathogens.. | 33 |
| <i>Draeculacephala mollipes</i> , gall
formation by | 41 | <i>Gymnosporangium juniperi-virgini-</i>
<i>anae</i> | 49 |
| <i>Drino bohémica</i> , host selection by .. | 23 | Gyptol | 69, 70 |
| Eclipic acid | 16 | Habrobracon venom, as insecticide | 68 |
| Eelworm hatching factor | 15 | <i>Hamamelis virginiana</i> | 41 |
| <i>Empoasca devastans</i> | 25 | <i>Helianthemum lavandulaefolium</i> .. | 12 |
| <i>Empoasca faciatis</i> | 25 | <i>Helminthosporium sativum</i> , toxins
from | 44 |
| <i>Encelia farinosa</i> , phytotoxins
from | 12, 13 | <i>Heterodera cruciferae</i> | 15 |
| <i>Endothia parasitica</i> , toxins from .. | 44 | <i>Heterodera rostochiensis</i> | 15 |
| Enzyme induction | 81 | <i>Heterodera schachtii</i> | 15, 16 |
| <i>Ephestia kuehniella</i> | 23 | <i>trans</i> -2-Hexenal | 69 |
| <i>Erica multiflora</i> | 12 | <i>Hieracium pilosella</i> | 12 |
| <i>Escherichia coli</i> | 77 | Higher plants, toxins from .. | 9 et seq. |
| 2-Ethyl-1,4-dihydroxybenzene from
insects | 69 | <i>Holcus mollis</i> | 9 |
| 2-Ethyl-1,3-hexanediol, as insect
repellent | 25 | Homeostat | 82 |
| Ethylene, phytotoxicity of | 49 | Hopkins Host Selection Principle | 22, 68 |
| Ethylene chlorohydrin, effect on
dormancy | 17 | <i>Hormaphis hamamelidis</i> | 41 |
| <i>Euproctes chrysorrhæa</i> , chemotrop-
ism of | 24 | Host resistance, to fungi | 33 et seq. |
| <i>Eurosta solidaginis</i> | 42 | Host resistance, to insects.. | 22 et seq. |
| <i>Eurycotes florindana</i> , insecticide
from | 69 | Hydrogen cyanide, as phytotoxin.. | 14 |
| Flax, wilt resistance of | 14 | Hydrogen cyanide, from millipedes | 69 |
| "Florigen" | 28 | Hydrojugone | 12 |
| Fluctuation tests | 80 | Hydroxylamine, virus induction by | 79 |
| Fluvomycin | 77 | 5-Hydroxy-1,4-naphthoquinone, as
phytotoxin | 12 |
| <i>Franseria dumosa</i> | 12, 13 | Indoleacetic acid | 13, 17, 49, 50, 73 |
| Furocoumarins, as phytotoxins ... | 12 | Indolylacetonitrile | 17 |
| Fusaric acid | 45 | Insect repellents | 25 |
| Fusarinic acid | 45 | Iridomyrmecin | 67 |
| | | Japanese beetle, host selection by .. | 23 |
| | | <i>Juglans cinerea</i> | 11 |
| | | <i>Juglans nigra</i> | 11 |
| | | Juglone, as phytotoxin | 12 |
| | | Kataplasmic galls | 41 |
| | | Latent virus | 79 |
| | | <i>Latheticus oryzae</i> , insecticides from | 69 |
| | | <i>Leptinotarsa decemlineata</i> , resist-
ance to | 25, 26 |

SUBJECT INDEX

<i>Sorghum vulgare</i>	15	Transforming agents	84
Soybean, plowing in of	60	<i>Tribolium castaneum</i>	69
Soybean, resistance to insects of ..	26	<i>Tribolium confusum</i>	69
Soyin	26	<i>Tribolium destructor</i>	69
<i>Spergula arvensis</i>	9	<i>Trichoderma lignorum</i>	54
Spurrey, effects on crop plants	9	<i>Trichoderma viride</i>	14, 54, 61
<i>Stellaria media</i>	9	<i>Trichogramma evascens</i>	25
<i>Streptomyces griseus</i>	58	Trichothecin	59, 60
<i>Streptomyces rimosus</i>	62	<i>Trichothecium roseum</i>	59
<i>Streptomyces venezuelae</i>	60	Trimethylethylene, as fungicide ...	43
Streptomycin	62, 63	<i>Trionymus sacchari</i>	41
<i>Striga hermonthica</i>	15	Tropolone derivatives, as fungicides	35
<i>Striga lutea</i>	15	Tumor-inducing principle	73, 84
Subtilins	77	Twitch grasses, effects on crop	
Swine cress, in eelworm control ...	16	plants	9
Systemic fungicides	62	Tyrocidines	77
Tabtoxinine	75	<i>Uromyces pisi</i>	49
Tartarian lamb	11	<i>Ustilago hordei</i>	62
Télétoxie	12	<i>Ustilago nuda</i>	62
Tetrahydroxystilbene, as fungicide.	36	<i>Ustilago zeae</i>	49
<i>Thamnosa montana</i>	12	Vanillin, as phytotoxin	8
Thiamine, from plant roots	38	<i>Verticillium albo-atrum</i>	48
<i>Thuja plicata</i>	35	Viridin	56
Thujaplicins, as fungicides	35	Vivotoxins	43
Tobacco mosaic, strains of	82	Virus, structure of	82
Tobacco, "wild fire"	74	Walnuts, effects on vegetation	11
Tomatin	26, 34	"Wildfire" toxin	74
Tomato moth, photoperiodism in ...	28	Wireworms, food finding by	24
<i>Toxylon pomiferum</i>	36	Wormwood, phytotoxins of	13
<i>Trachypogon plumosus</i>	13	D-Xyloketose	15
Transduction	84		

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